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THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

# EXPERIMENTS EMPLOYING A QUANTITATIVE METHOD IN A STUDY OF THE D'HÉRELLE PHENOMENON.

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*From the Department of Pathology and Bacteriology in the University of Toronto.*

Forwarded for publication March 28th, 1922.

THE phenomenon of d'Hérelle consists, in general terms, of changes in bacterial cultures initiated by some substance of obscure nature that has been obtained from most diverse sources. One of the striking changes is lysis, indicated by clearing of a bouillon culture. Lysis can be reproduced in series, thus giving the impression that the substance causing it has the power of growth. The exact nature of this substance is a controversial point, and a review of the literature has been published in another place (Maitland, 1922).

Throughout this paper the substance will be termed the "active substance." A quantitative method for estimating the strength of a sample of active substance is described. It is pointed out that increase of active substance in a bouillon culture can occur without lysis; in fact it appears to bear no relation to changes in bacterial content of the culture in which it is increasing. The quantitative method is also applied to the elucidation of other problems.

## PREPARATION OF SAMPLES OF ACTIVE SUBSTANCE.

Seven samples were isolated from fæces of hospital patients. Four had infection with *B. paratyphosus B*, two with *B. typhosus*, and one had acute appendicitis followed by portal pyelophlebitis and liver abscesses which yielded *B. mucosus capsulatus*.

The technique was to add about 0·5 c.c. fæces to 50 c.c. bouillon (pH 7·8–8·0) and incubate at 37° C. overnight. The culture was filtered through infusorial earth and a Mandler filter. One cubic centimeter of this filtrate added to a young bouillon culture of an appropriate organism resulted in the appearance of active substance. All samples acted on several organisms and produced typical effects in successive cultures.

Table I shows the origin of the samples, the organisms acted upon, and indicates in a general way the amount of action.

TABLE I.

| Name of patient. | | Timbers. | Collins. | Aitken. | Rotz. | Gibson. | Walker. | McPhee. |
|---|---|---|---|---|---|---|---|---|
| Patient's infection | | *B. paratyphosus B.* | *B. paratyphosus B.* | Appendicitis. Liver abscesses. | *B. paratyphosus B.* | *B. paratyphosus B.* | *B. typhosus B.* | *B. typhosus.* |
| Organisms acted upon | *B. coli* | 0 | 0 | 0 | + | 0 | 0 | 0 |
| | *B. paratyphosus A.* | + | + | + | + | 0 | 0 | 0 |
| | *B. paratyphosus B.* | ++ | ++ | ++ | ++ | 0 | + | + |
| | *B. dysenteriæ* | ++ | ++ | ++ | ++ | + | + | + |
| | *B. typhosus* | 0 | 0 | 0 | + | 0 | 0 | 0 |

The organisms acted upon were all from stock cultures, and were the only ones employed for the experiments of this paper.

## MEASUREMENT OF THE STRENGTH OF A SAMPLE OF ACTIVE SUBSTANCE.

The method of measurement devised for these experiments is based on the graded appearances illustrated in Fig. 1.

When the strength of any sample is to be determined six tubes are set up. The first contains active substance, the others bouillon. A dilution of 1 in 10 is made in the second tube, 1 in 100 in the third, continuing to 1 in 100,000 in the sixth. Six agar slants are inoculated, each with one loopful from a tube of active substance and a loopful of a young bouillon culture of the organism in association with which that sample of active substance had been produced. After incubation overnight the strength of the sample will be indicated by comparing each tube with the graded series in Fig. 1. For convenience in recording results the gradations have been designated by numbers. Thus the strength of a sample might be indicated by 8 7 6 4 2 1.

The method described does not depend on any one dilution, but takes into account all six tubes. It does not depend upon any theory as to the nature of active substance. It has given consistent readings with the same sample. The expression of strength is not absolute, but it has proved to be consistently comparative. It seems to be, therefore, the nearest approach to accurate measurement that can be obtained, in the absence of exact knowledge as to the nature of the substance being measured. As such it has been used to make a quantitative study of some aspects of the phenomenon under discussion.

## THE RATE OF INCREASE OF ACTIVE SUBSTANCE IN BOUILLON CULTURES.

For the investigation of this point active substance derived from Timbers and *B. dysenteriæ* was employed.

To each of several identical tubes of young bouillon culture was added an amount of active substance known by previous determination to give a reading of about 3 0 0 0 0 0. The tubes were placed at 37° C. From time to time one was filtered through a Mandler filter and the strength of the filtrate measured. Thus a number of readings were obtained giving the strength of samples at various times. A control of active substance in bouillon in every instance showed no increase.

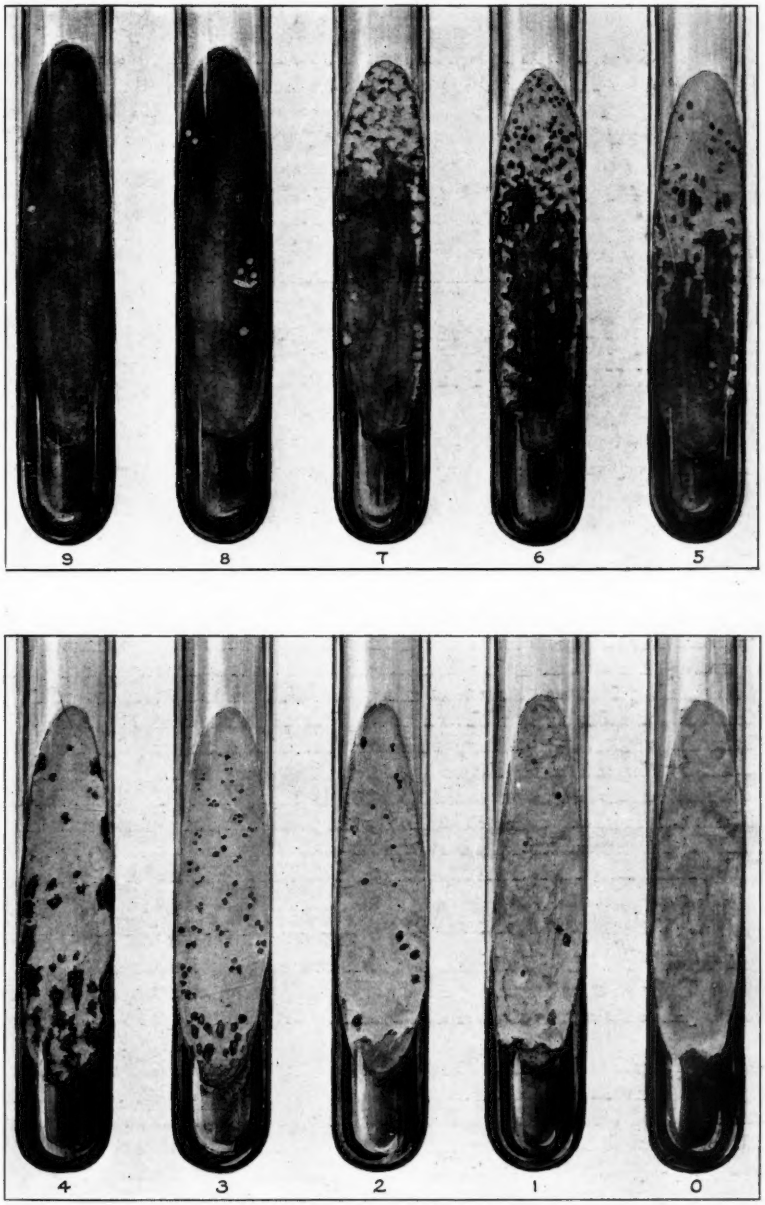


FIG. 1.

Graded appearances on agar slants inoculated with *B. dysenteriæ* and falling doses of active substance after 24 hours' incubation. Tube No. 9, showing complete inhibition of growth, corresponds to a full strength sample of active substance, and the decreasing numbers to progressively increasing dilutions. In tube No. 0 the dilution is so great that the bacterial growth appears normal. All tubes were made from the same sample of active substance and *B. dysenteriæ*.

Some results are given to illustrate this increase. Timbers' active substance was transferred in series on *B. dysenteriæ*, and therefore had always acted on that organism.

TWO-HOUR CULTURE.

| Time after addition of active substance. | Readings. | | | | | |
|---|---|---|---|---|---|---|
| 10 min. . . | 2 | 0 | 0 | 0 | 0 | 0 |
| 1 hr. . . | 3 | 1 | 0 | 0 | 0 | 0 |
| 2 hrs. . . | 7 | 6 | 5 | 1 | 1 | 1 |
| 3 hrs. 45 min. | 8 | 8 | 6 | 4 | 2 | 1 |
| 5 hrs. 45 min. | 9 | 8 | 8 | 5 | 4 | 1 |
| 7 hrs. . . | 8 | 8 | 7 | 5 | 3 | 1 |
| 22 hrs. 30 min. | 9 | 8 | 8 | 7 | 6 | 3 |
| 49 hrs. 30 min. | 9 | 8 | 7 | 7 | 3 | 1 |
| 9 days . . | 9 | 9 | 8 | 6 | 6 | 1 |

TWENTY-HOUR CULTURE.

| Time after addition of active substance. | Readings. | | | | | |
|---|---|---|---|---|---|---|
| 10 min. . . | 1 | 1 | 0 | 0 | 0 | 0 |
| 1 hr. . . | 1 | 0 | 0 | 0 | 0 | 0 |
| 2 hrs. . . | 2 | 0 | 0 | 0 | 0 | 0 |
| 3 hrs. 45 min. . | 6 | 5 | 1 | 1 | 0 | 0 |
| 5 hrs. 30 min. . | 8 | 7 | 5 | 3 | 1 | 1 |
| 8 hrs. 15 min. . | 8 | 8 | 4 | 1 | 1 | 0 |
| 11 hrs. 45 min. | 8 | 8 | 4 | 3 | 1 | 1 |
| 25 hrs. 30 min. | 8 | 8 | 7 | 5 | 3 | 1 |
| 7 days . . | 8 | 8 | 6 | 5 | 2 | 1 |

TWENTY-FOUR-HOUR CULTURE.

| Time after addition of active substance. | Readings. | | | | | |
|---|---|---|---|---|---|---|
| 1 hr. 10 min. . . . | 2 | 0 | 0 | 0 | 0 | 0 |
| 3 hrs. 30 min. . . . | 8 | 8 | 6 | 5 | 2 | 1 |
| 5 hrs. . . . . | 9 | 7 | 5 | 4 | 2 | 1 |
| 7 hrs. 30 min. . . . | 8 | 8 | 6 | 4 | 1 | 0 |
| 4 days . . . . | 8 | 8 | 7 | 4 | 1 | 1 |

These readings demonstrated that increase does occur. The method affords, therefore, a means of detecting changes in strength of active substance in any experimental circumstances.

It was also shown that the curve of increase had three phases. During the first hour, or occasionally longer, there was no increase. This was followed by a period of rapid increase. Generally this occupied the second and third hours, during which the active substance increased to about ten thousand times its original strength. During the fourth hour the rate of increase slackened. By the fifth hour it appeared to be established at its maximum. From that time the curve entered the third phase of equilibrium, which lasted as long as readings were made, up to nine days.

## FACTORS AFFECTING THE INCREASE OF ACTIVE SUBSTANCE IN BOUILLON CULTURES.

It was found by measurement that active substance did not increase in a saline suspension of living bacteria, from an eighteen-hour bouillon culture, washed free from all growth products and accessory substances. Timbers' active substance and *B. dysenteriæ* were used. It was evident therefore that three factors were necessary for increase: (1) a small amount of active substance itself, (2) living bacteria, (3) some accessory factor.

Various substances were then tested to determine if they contained an accessory factor. Small amounts were added to active substance in a saline suspension of washed bacteria and the method of measurement applied to detect increase.

It was found that bouillon (prepared with "Difco" peptone) itself acted as an accessory factor and that the minimum effective amount lay somewhere between 1 per cent. and 0·1 per cent. Active substance did not, however, increase in bouillon alone.

The following readings serve to illustrate:

*B. dysenteriæ from a 24-hour Bouillon Culture: Timbers' Active Substance.*

| | Time after addition of active substance. | Readings. | | | | | |
|---|---|---|---|---|---|---|---|
| Saline suspension + 5 per cent. bouillon | 5 hrs. | 8 | 7 | 6 | 2 | 0 | 0 |
| | 8 hrs. | 8 | 6 | 6 | 3 | 2 | 0 |
| | 24 hrs. | 9 | 7 | 5 | 5 | 2 | 0 |
| Saline suspension + 1 per cent. bouillon | 5 hrs. | 6 | 3 | 2 | 0 | 0 | 0 |
| | 8 hrs. | 8 | 7 | 4 | 2 | 2 | 0 |
| | 24 hrs. | 8 | 7 | 5 | 4 | 1 | 0 |
| Saline suspension + 0·1 per cent. bouillon | 5 hrs. | 2 | 1 | 0 | 0 | 0 | 0 |
| | 8 hrs. | 2 | 0 | 0 | 0 | 0 | 0 |
| | 24 hrs. | 2 | 0 | 0 | 0 | 0 | 0 |
| Control, active substance + saline suspension alone | 24 hrs. | 2 | 1 | 0 | 0 | 0 | 0 |

The addition of bacterial growth products to bouillon did not alter its effectiveness. That is to say, a Berkefeld filtrate of an eighteen-hour bouillon culture of *B. dysenteriæ* had the same effect as bouillon alone.

Meat infusion prepared as in making bouillon but without salt or peptone gave the same result as bouillon. Hæmoglobin appeared not to be essential. Extracts were made of sheep's erythrocytes and defibinated sheep's blood, but no active preparation was obtained. The extracts were made by adding 2·5 per cent. washed erythrocytes or 5 per cent. of defibrinated blood to distilled water. The mixtures were heated at 50° C. for 30 minutes, and boiled for 15 minutes. At this stage they were inactive. These permanently turbid solutions were then precipitated by adding NaCl. The supernatant fluid was autoclaved at 15 lb. for 30 minutes and gave a yellowish tinged fluid that was also inactive.

Ascitic fluid free from hæmoglobin was effective, as was also human citrated plasma.

The third factor was also found in potato. A piece from the centre of a potato was removed aseptically, minced, and extracted in normal saline for 36 hours or longer. This extract, bacteria free, permitted increase.

## THE RELATION OF THE CURVE OF INCREASE OF ACTIVE SUBSTANCE IN A BOUILLON CULTURE TO VARIATIONS IN THE NUMBER OF ORGANISMS.

If increase in active substance depends on bacterial division it should be possible to demonstrate some relation between this increase and the bacterial increase. One cannot, however, use the growth rate in a culture that contains active substance on account of the factors of lysis and inhibition. Just how these might operate would be difficult to predict; the observation of many

cultures suggests that it is not always in the same manner. In some cases increase is accompanied by clearing or by inhibition, but there may be increase with very little change in the bacterial content.

It can be demonstrated that the curve of increase has no apparent relation to the growth curve in a parallel culture without active substance. Employing a saline suspension of washed living bacteria plus 1 per cent. bouillon there was increase according to the readings cited, but the bacteria in a parallel tube at most doubled in number. The method described by Gates (1920) was used to estimate the number of organisms. It was found, also, that active substance increased equally in four- to six-hour cultures and in twenty-four-hour cultures, in which the growth rate was dissimilar at the time active substance was added.

Therefore it appears that active substance can increase some ten thousand-fold while the number of bacteria in a parallel culture is doubling. There is no reason for supposing that growth would be more rapid in the tube with active substance. On the other hand the effect of active substance may be quite complex, and variation of the total bacterial content may be the sum of destruction, inhibition and stimulation proceeding simultaneously. It is possible also that bacterial metabolism may be affected independently of bacterial division. Although the absence of apparent relation between increase and the growth curve in a parallel culture is of interest in itself, it does not essentially advance the problem of the interaction of active substance and bacteria. The fact that increase may coincide with varying changes in the total bacterial content of the culture suggests that the interaction is probably the sum of several effects proceeding simultaneously.

### THE EFFECT ON THE LYTIC AND INHIBITING QUALITIES OF ACTIVE SUBSTANCE OF THE ORGANISM IN ASSOCIATION WITH WHICH IT IS PRODUCED.

If increase of active substance is the result of bacterial metabolism, and since increase will occur with more than one organism, it might be supposed that the qualities of a sample would be influenced by the organism in association with which it is produced.

This possibility was examined by the following experiment. A filtrate from a culture of fæces (Walker) was found to yield active substance when added to *B. dysenteriæ* and *B. paratyphosus B*. The quality of the sample obtained by filtering the first culture of *B. dysenteriæ* was subjected to the method of measurement with two organisms, *B. dysenteriæ* and *B. paratyphosus B*. This sample was allowed to increase through twelve cultures of *B. dysenteriæ* and measured again. If any change in the lytic or inhibiting qualities had occurred there should have been some difference in the readings. All the readings were, however, identical.

Similarly, the filtrate from the first culture of *B. paratyphosus B* was passed through twelve cultures of *B. paratyphosus B* and measured with both organisms before and after. All these readings were identical. Also, they were identical with those of the other sample.

Thus the lytic and inhibiting qualities of a freshly isolated sample of active substance were not altered by allowing it to increase repeatedly in association with different organisms. If these organisms actually did produce the increase

then *B. dysenteriæ* and *B. paratyphosus B* must each have produced a sample with the same qualities.

## CONCLUSIONS.

The rate and amount of increase of active substance in a culture can be measured.

The curve of increase has three phases, an early period of very slight increase, a comparatively short period of rapid production, and a stationary period commencing when an equilibrium has been reach at about five hours and lasting for at least nine days.

Three factors are necessary for increase: (1) A small amount of active substance, (2) living bacteria, (3) an additional factor.

The increase of active substance in a bouillon culture does not coincide with any constant alteration in the bacterial content of the culture in which it is being produced. Clearing does not always occur, and there may be increase in the total number of bacteria.

The lytic and inhibiting qualities of a sample of active substance were not altered by causing it to increase repeatedly in association with two different organisms. If the organisms themselves produced the active substance then each (*B. dysenteriæ* and *B. paratyphosus B*) must have produced a sample with the same qualities.

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FIG. 1.

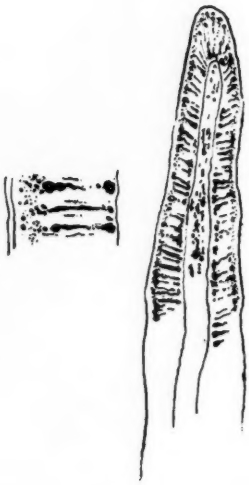

FIG. 2.

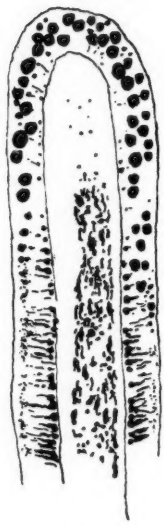

FIG. 3.

Low power camera lucida drawings of villi stained with osmic acid. Fig. 1 shows absorption by drops. From a rat fed on a vitamin-free diet. Fig. 2 shows absorption by streams. From a rat fed on a diet containing vitamins A and B. Fig. 3 shows absorption by drops at the apex of the villi and streams at the base. From a rat fed on a diet containing vitamin B, but not A.

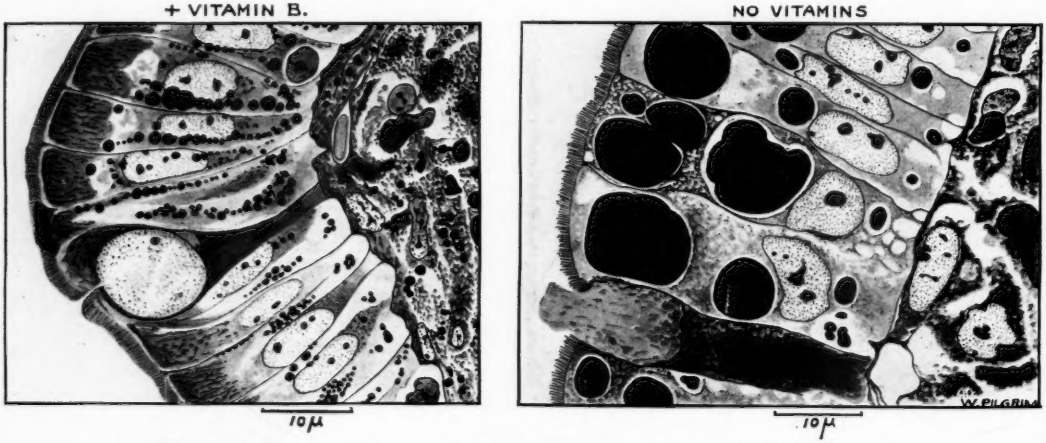


FIG. 4.—High-power camera lucida drawing of intestinal epithelium (duodenum) of two rats. The one had eceived a vitamin-free diet, the other a diet containing vitamin B.

Mottram, Cramer and Drew. Fat Absorption.

# VITAMINS, EXPOSURE TO RADIUM AND INTESTINAL FAT ABSORPTION.

J. C. MOTTRAM, W. CRAMER, AND A. H. DREW.

*From the Radium Institute and from the Imperial Cancer Research Fund, London.*

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NUMEROUS descriptions and figures are given in the literature of the histological appearances of the absorption of fat by the epithelial cells of the small intestine in mammals. They all show as the first stage an appearance of droplets of fat between the free border and the nucleus. These increase in size and may fill the whole cell. How these fat-droplets leave the cell again is a question which has given rise to a good deal of discussion, but no satisfactory solution of the problem has as yet been found. After they have left the epithelial cell the fat-droplets find their way into the central lacteal of the villi, and in this transport the lymphocytes, according to Schafer (1912), play an important part. We shall call this mode of fat absorption "absorption by drops." Schafer also states that puppies and kittens fed on milk show a different type of fat absorption: "darkly stained streaks may be seen (in osmic preparations) extending from the interepithelial spaces to the borders of the central lacteal." This type of fat absorption when the fat is in a much more finely divided form we shall call "absorption by streams." So far as we have been able to find, it is mentioned only briefly in Schafer's text-book and no figure of it appears to exist. The conditions governing the two types of fat absorption are not known. During the study of the lymphoid atrophy following radiation and vitamin B deficiency it was found that these two types of fat absorption can be produced at will in the same animal. The object of the present paper is to report the main facts observed.

Rats were the animals experimented upon. They were kept without food for 20 hours. They were then fed artificially at a given time by passing a narrow glass tube down the throat through which the food was introduced. The animals to be compared were fed at the same time and killed some hours later at the same time. The number of hours varied in different experiments from $2\frac{1}{2}$ hours to 7 hours. Pieces of different parts of the small intestine exactly equidistant from the pylorus were cut out, fixed in formalin, and then transferred to a solution of 1 per cent. osmic acid in 1 per cent. chromic acid. The tissue was examined in paraffin section.

In the first set of experiments two batches of animals were fed, the control set on standard diet and olive oil, the other set B with standard diet, olive oil and vitamin B, given in the form of "marmite."

The standard vitamin-free diet consisted of starch, purified casein and salt mixture.

In set B in which vitamin B was present the fat was absorbed by streams, in some cases by drops at the apices of the villi and by streams at their sides. In the control set which had no vitamin the fat was absorbed entirely by drops. If the fat is absorbed by streams the central part of the cell between the nucleus, which is usually centrally placed, and the free border is free from fat-droplets, and a stream of very finely divided fat-droplets is seen running through the cell on either side of the nucleus. Fine fat-droplets are also present in abundance in the reticular tissue in the centre of the villi. We wish to emphasize that the stream of droplets in our preparations is within the epithelial cell: we have not seen the passage of fat-droplets between the cells described by Reuter (1902). If absorption is by drops, large globules of fat accumulate in the central part of the cell between the free border and the nucleus, which is frequently pushed towards the basement membrane. Very few fat-droplets are to be seen in the reticular tissue in the centre of the villus, and when present they are relatively large. The difference between the two appearances is so striking that a glance with the low power at the preparation enables one to tell with certainty whether the animal had been fed on a vitamin-rich diet or a diet free from vitamins.

In a second set of experiments cod-liver oil was used instead of olive oil, so that the animals received an ample supply of vitamin A. When vitamin B was added the fat was absorbed entirely in the form of streams. When vitamin B was omitted and only the fat-soluble vitamin A was present the absorption took place in drops at the apex and a few streams at the sides. It should be specially noted that these effects were observed in normal rats which had not been subjected to any vitamin deficiency previous to these experiments. The effect on fat absorption is therefore an immediate one.

The effect of radiating animals was next investigated. In order to determine how the great reduction in the number of circulating lymphocytes affects fat absorption, animals received a large dose (0·66 rads) of screened radiation (0·1 mm. lead). No fat absorption at all occurred, even in the presence of vitamin B and A. When 0·28 rads was given, only absorption by drops occurred whether or not vitamins were present. When 0·09 rads was the dose applied, then olive oil with vitamin B gave only drops, and cod-liver oil containing vitamin A with the further addition of vitamin B only drops and slight streams at the bases of the villi.

That almost all previous observers have found only the fat absorption by drops explains itself readily from the fact that when details are given the fat used for feeding was either bacon fat or lard or olive oil. These materials are free from the water-soluble vitamin B and contain little if any fat-soluble vitamin A. It is significant that the absorption of fat by streams described by Schafer was seen in animals fed on milk which contains both vitamins. We have noted that in the rats receiving a vitamin-free meal relatively large masses of fat are sometimes seen lying within the lumen of the intestine, between the opposing villi. This suggests that the digestion of fat is less complete or less rapid in the absence of vitamins, and that vitamins may have a stimulating action on the digestive processes. Voegtlin and Myers (1919)

have shown that food rich in vitamin B contains substances which stimulate the secretion of bile and of pancreatic juice. And although their further contention that vitamin B and secretin are identical has been disproved by the work of Anrep and Drummond (1921), the actual observations of the last-named authors confirm the important and significant fact that the activity of the liver and pancreas can be stimulated by the presence of vitamin B in the food.

The effect of radiation is a striking confirmation of Schafer's views on the importance of the lymphocytes in fat absorption. In fact their function appears to be even more important than the mere transport of fat which he assigned to them. Their presence or absence seems to determine the physiological condition of the epithelial cells even more powerfully than the presence of vitamins.

There is, as our figures show, an essential difference with reference to the functional activity of food absorption between the state of a cell absorbing fat by drops, and the state of a cell where a stream of very finely divided fat runs through the cell on either side of the nucleus as if the cell were canalised.

The problem how exactly these various facts are related to each other and what secondary effects they may produce in the bacterial flora of the intestine is a matter for further investigation. One factor we can definitely exclude: experiments with coloured food show that the rate of passage of food through the stomach and small intestine is not delayed at once when vitamins are withheld from the food. There is no intestinal stasis in that sense. A deficient absorption of fat is in itself not an adequate explanation of the marasmus which results from a deficiency of the water-soluble vitamin or from exposure to radium, since animals can live on a diet free from fat. But if we take the difference in the absorption of fat described in this paper as an indicator of difference in the functional state of the intestinal epithelium in regard to the absorption of food, we can draw the general conclusion that the vitamins A and B, particularly the latter, produce a definite stimulating action on the processes of intestinal digestion and absorption. The presence of lymphocytes in the intestine is an even more important factor. This confirms the conclusion expressed by us in a previous paper (Cramer, Drew and Mottram, 1921), that the marasmus resulting from withholding the water-soluble vitamin from the diet is due to an impaired absorption and assimilation of food from the intestine.

The radium used in this investigation was a loan from the Medical Research Council.

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# INDICATORS FOR CULTURE MEDIA CONTAINING VARYING ACIDS AND BUFFERS.

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WHEN the final pH of a bacterial culture is ascertained, the determination has often to be made in the presence of various acids produced during growth, such as lactic, acetic, succinic, etc. Similarly, in estimating the pH values of pathological fluids associated with infective or other agents, the dissociation products and buffer influences of carbonic, sulphuric, acetic and phosphoric acids have to be taken into account.

During an investigation in which dilute solutions of typical examples of the various types of acids were introduced into culture media prior to insemination with bacteria, it was recognised that a wider experience with colour indicators was called for, more especially with regard to the behaviour of the indicators in mixtures containing neutral salts or solutions of weak acids.

Discussing the problems involved with Prof. J. W. McBain, of our University Department of Physical Chemistry, I was offered the services of one of his "Honours" students, Miss M. Kieser, for the control of our colorimetric estimations by E.M.F. determinations. She has carried out this part of the work with great care, and is responsible for the electrical measurements.

In addition to the object of ascertaining the limits of colorimetric work, it was considered worth while to examine in detail the buffer content of the media employed.

## METHOD.

A large batch of casein sugar-free broth (Hall, 1918) or other medium was prepared. This was made neutral to phenol-phthalein, and then brought to the required pH by the addition of the necessary amounts of the several normal acid solutions, and a colorimetric estimation made. Immediately this had been done, a sample was taken by Miss Kieser, and the E.M.F. determined. After the E.M.F. reading was taken, an additional colour test was carried out.

The acidity and alkalinity reserve was ascertained by a method similar to that of Brown (1921) and the buffer index calculated.

## BUFFER CONTENTS.

The variations of the buffer factors in different media cannot be neglected, especially when a rapid or large production of acidity is aimed at, or when it

is desired that the final pH shall be low. Some of our composite media protect bacterial substance from acids or salts, as for instance by the additions of calcium or other substances, while others fail to do so, and consequently are associated with less active metabolism and growth. This absorption or abstraction may also decrease or augment the differential manifestations of a bacterium, and accordingly alter its specific capacities. Similarly in making use of the action of cation, anion, osmotic or dissociation influences during growth, it is an advantage to standardise the reserve alkalinity and acidity, that is to say, the buffer index, of each batch of medium employed.

By this means the micro-organisms are provided with more stable conditions for developing their fullest activities and imitation of the "*in vivo*" surroundings is more nearly attained.

Quite apart from the question of accelerating or inhibiting the growth of particular organisms, Brown (1921) suggests that a medium with a low buffer index should be selected when rapid indications of fermentation are required, and one of high reserve alkalinity and buffer index when abundant growth and large acid production are desired. As a guide, therefore, the contents of Table I are submitted. The average amount of N/10 acid necessary to adjust the medium from phenolphthalein to phenol red neutrality is indicated in brackets as dilutions.

Thus $HNO_3$ in the veal broth represents a dilution of N/10 $HNO_3$ in the total fluid medium of 1 in 14, in peptone 1 in 12, etc.

TABLE I.—*Comparative Buffer Indices of Media pH 7·6 and Equal Amino-Acids containing Dilute Acids.*

| Acids. | Dissociation per cent. at 18°–25°C. | Lait-proto broth 1·8. | Veal broth 2·2. | Lait-proto broth + 1 per cent. peptone 1·9. | Veal broth + 1 per cent. peptone 2·55. | Bacto-peptone 3·3 per cent. 2·85. | Bacto-peptone 1·2 per cent. 1·95. | Meat extract 2·6. |
|---|---|---|---|---|---|---|---|---|
| **Mineral:** | | | | | | | | |
| $HNO_3$ | 94 | 1·9(14) | 2·4(14) | 1·95(14) | 2·7(13) | 3·1(12) | 2·2(12) | 2·8(13) |
| HCl | 91 | 1·85(14) | 2·3(14) | 1·95(14) | 2·7(13) | 3·0(13) | 2·15(13) | 2·8(14) |
| $H_2SO_4$ | 62 | 1·85(14) | 2·5(14) | 2·0(14) | 2·8(13) | 3·2(12) | 2·2(12) | 2·6(13) |
| Phosphoric | 13·9 | 1·8(8) | 2·4(8) | 1·9(8) | 2·6(8) | 2·8(7) | 1·8(7) | 2·6(8) |
| **Sat. monobasic:** | | | | | | | | |
| Formic | 4·5 | 1·95(14) | 2·5(14) | 1·9(14) | 2·7(13) | 3·0(12) | 2·2(12) | 2·7(14) |
| Acetic | 1·3 | 2·0(14) | 2·5(14) | 2·0(14) | 2·9(13) | 3·3(12) | 2·2(12) | 2·8(13) |
| Propionic | 1·2 | 1·9(14) | 2·4(15) | 2·0(14) | 2·8(13) | 3·1(12) | 2·1(12) | 2·7(13) |
| Butyric | 1·1 | 1·95(14) | 2·4(14) | 1·9(14) | 2·8(13) | 3·1(12) | 2·1(12) | 2·7(13) |
| **Sat. dibasic:** | | | | | | | | |
| Oxalic | 9·0 | 2·0(14) | 2·5(14) | 2·0(14) | 2·9(13) | 3·0(12) | 2·1(12) | 2·7(13) |
| **Monobasic hydroxy:** | | | | | | | | |
| Lactic | 3·6 | 1·95(14) | 2·4(14) | 2·0(14) | 2·8(13) | 3·1(12) | 2·2(12) | 2·7(13) |
| **Dibasic dihydroxy:** | | | | | | | | |
| Tartaric | 13·9 | 1·95(14) | 2·4(15) | 2·0(14) | 2·8(13) | 3·0(12) | 2·1(12) | 2·7(13) |
| **Tribasic hydroxy:** | | | | | | | | |
| Citric | 15·2 | 1·9(14) | 2·4(13) | 2·0(14) | 2·7(13) | 3·0(12) | 2·1(12) | 2·7(13) |

The findings show a slight buffer increase in the media containing the added acids; this is probably associated with the liberation of anions.

The effects of sterilisation and storage upon the buffer contents call also for inquiry. Taking all the findings into consideration, it may be stated that the changes are minimal so far as bacteriological requirements are concerned. An illustration is appended in Table II, the pH 6·8 being selected as a contrast to the 7·6 of Table I.

TABLE II.—*Effects of Storage on Buffer Indices in the Presence of Various Acids.*

Peptone water (amino-acid 40) (Hall, 1918).

| pH 6·8 adjusted with— | Before sterilisation. | After sterilisation. | Stored at 17° C. for 26 days. |
|---|---|---|---|
| $HNO_3$ | 2·25 | 2·37 | 2·37 |
| HCl | 2·4 | 2·35 | 2·35 |
| $H_2SO_4$ | 2·6 | 2·54 | 2·54 |
| Phosphoric | 5·0 | 4·89 | 4·89 |
| Formic | 2·45 | 2·38 | 2·38 |
| Acetic | 3·25 | 3·18 | 3·16 |
| Propionic | 2·75 | 2·68 | 2·68 |
| Butyric | 2·9 | 2·86 | 2·85 |
| Oxalic | 2·75 | 2·67 | 2·65 |
| Lactic | 2·0 | 1·96 | 1·96 |
| Tartaric | 2·37 | 2·37 | 2·37 |
| Citric | 2·75 | 2·74 | 2·73 |

It is common practice to add fresh blood, serum, tissue fluids or tissues to media in order to induce profuse bacterial growth. Frequently as much as 10 per cent. or more is added. McLeod and Wyon (1921) have shown that the growth-promoting power does not bear any ratio to the known vitamine contents, and suggest that it is a phenomenon of the colloid state. Whether it is in any way due to buffer changes seems worth the inquiry. Table III contains a few of the estimations to show that the buffer alteration is very slight when blood is employed and when the added acids are in such dilutions as fail to yield more than the lower limits of protein-acid combination, or to augment the viscosity. But both Tables II and III indicate the advantages to be gained by adjusting the various media used in a laboratory to a similar buffer index for purposes of comparative growths, and for maintenance of stock cultures. Equally, the preparation of media with buffer content suitable for rapid fermentation or slowed growth is evident.

TABLE III.—*Effect of Blood on Buffer Indices.*

| | pH. | Reserve | | Buffer index. |
|---|---|---|---|---|
| | | Acidity. | Alkalinity. | |
| Lait-proto broth | 7·7 | 0·7 | 2·0 | 2·7 |
| Lait-proto broth + 1/10 Blood | 7·6 | 0·75 | 2·25 | 3·0 |
| Lait-proto broth + 1/10 Blood + 1/100 $HNO_3$ | 7·3 | 0·90 | 2·0 | 2·9 |
| Lait-proto broth + 1/10 Blood + 1/200 $HNO_3$ | 7·5 | 0·80 | 2·4 | 3·2 |
| Lait-proto broth + 1/10 Blood + 1/100 lactic | 7·3 | 0·90 | 2·0 | 2·9 |
| Lait-proto broth + 1/10 Blood + 1/200 lactic | 7·4 | 0·80 | 2·2 | 3·0 |

## EXTENDED USE OF COLOUR INDICATORS.

The value of colorimetric readings is shown in Table IV. With practice it is possible to obtain close figures, even in the presence of all the types of acids employed. The results are, however, more likely to be less precise in mixtures when there are interacting substances and in the presence of sugars. The differences, nevertheless, are slight, and the error can be calculated.

Speaking generally, the E.M.F. finding is slightly in excess of the colour reading. This discrepancy may arise from a source similar to that which Evans (1921) describes in connection with the application of the E.M.F. to blood and other fluids containing carbonates or carbonic acid.

TABLE IV.—*Comparison of Colorimetric and E.M.F. Readings.*

Fluid media adjusted to 40 amino-acid and pH content with varying acids.

| | Lait-proto broth. | | | | | | Bacto-peptone. | | Lait-proto broth + bacto-peptone. | Lait-proto broth + bacto-peptone + 1 per cent. glucose. |
|---|---|---|---|---|---|---|---|---|---|---|
| | Colour. | E.M.F. | Colour. | E.M.F. | Colour. | E.M.F. | Colour. | E.M.F. | E.M.F. | E.M.F. |
| $HNO_3$ . . | 3·3 | 3·1 | 5·2 | 5·5 | 7·5 | 7·9 | 6·8 | 6·7 | 6·8 | 6·2 |
| HCl . . | 3·3 | 2·7 | 5·2 | 5·4 | 7·5 | 7·7 | 6·8 | 6·5 | 7·3 | 6·6 |
| $H_2SO_4$ . . | 3·3 | 2·9 | 5·2 | 5·4 | 8·0 | 8·0 | 6·8 | 6·8 | 6·6 | 6·7 |
| Phosphoric . | 3·3 | 3·5 | 5·2 | 5·6 | 7·5 | 7·4 | 6·8 | 6·8 | 6·8 | 6·7 |
| Formic . . | 3·3 | 3·4 | 5·2 | 5·6 | 7·5 | 7·7 | 6·8 | 6·7 | 7·1 | 6·3 |
| Acetic . . | 3·3 | 3·4 | 5·2 | 5·4 | 7·5 | 7·9 | 6·8 | 6·8 | 6·1 | 6·9 |
| Propionic . | 3·3 | 3·7 | 5·2 | 5·3 | 7·5 | 7·4 | 6·8 | 6·8 | 6·5 | 6·8 |
| Butyric. . | 3·3 | 3·8 | 5·2 | 5·4 | 7·5 | 7·7 | 6·8 | 6·6 | 6·8 | 6·9 |
| Oxalic . . | 3·3 | 2·5 | 5·2 | 5·5 | 7·5 | 7·5 | 6·8 | 6·8 | 6·9 | 6·9 |
| Lactic . . | 3·3 | 3·1 | 5·2 | 5·6 | 7·5 | 7·8 | 6·8 | 6·8 | 7·0 | 6·6 |
| Tartaric . | 3·3 | 3·1 | 5·2 | 5·7 | 7·5 | 7·6 | 6·8 | 6·6 | 7·1 | 6·5 |
| Citric . . | 3·3 | 3·0 | 5·2 | 5·4 | 7·5 | 7·7 | 6·8 | 6·8 | 6·9 | 6·7 |
| Indicator . | Brom-phenol blue | | Methyl red | | Phenol red | | Phenol red | | Phenol red | Phenol red |

## SUMMARY.

(1) Brom-phenol blue, methyl red and phenol red may be used as pH indicators for bacteriological media containing dilute quantities of certain typical mineral and organic acids.

(2) Buffer indices of media are slightly altered when small amounts of blood or of dilute acids are added. They are unaffected by storage.

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# VARIATION PHENOMENA IN STREPTOCOCCI, WITH SPECIAL REFERENCE TO COLONY FORM, HÆMOLYSIN-PRODUCTION, AND VIRULENCE.

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In spite of the numerous elaborate researches which have appeared periodically for many years past, no generally accepted method of classifying streptococci has yet been arrived at. Marmorek (1895) found that the morphological and growth characteristics were far too variable to allow either as a basis for grouping. Since Bordet (1897) found hæmolysed blood in the hearts of animals dying as a result of streptococcal infections, and Schotmüller (1903) made a distinction between hæmolytic and non-hæmolytic strains when grown on blood-agar, a broad classification into these two varieties has received fairly general acceptation. Each of the groups, however, is so large that attempts to subdivide them have occupied the attention of many workers in recent years.

One method used for this purpose has been the differentiation of strains according to their action in the fermentation of sugars. Gordon (1905) and also Andrewes and Horder (1906) found their results in this respect sufficiently constant to be of value, and Winslow (1912) came to the same conclusion. Broadhurst (1913) found that fermentation results varied according to the media employed, and while Holman (1916) divided streptococci on this basis, he did not believe that the grouping was directly correlated with disease production. Moreover, the constancy of these fermentation characters in streptococci has been called in question.

Agglutination by antiserum has been another method very frequently attempted for such a division. Early workers—Aronson (1903), Neufeld (1903)—found agglutination heterologous within the hæmolytic group, and Besredka (1904) agreed with their results. Some years later Floyd and Wolbach (1913) noted some evidence of such a grouping, but Kligler (1915) found no clear-cut division by agglutination methods. However, more recently, Avery, Dochez and Lancefield (1919), Havens (1919), Tunnicliff (1920) and Bliss (1920) have obtained much more encouraging results.

## SCOPE OF PRESENT RESEARCH.

This work was undertaken in an attempt to obtain evidence for or against the possibility of classifying streptococci by means of agglutination. From the beginning numerous hæmolytic strains gave difficulty because of spontaneous agglutination, and as time went on stable suspensions of non-hæmolytic strains became increasingly more difficult to obtain. Various media, solid and fluid, with varying pH and serum-content were tried, in the hope of finding conditions which would consistently be favourable to a growth which could be used for agglutination purposes. The results, however, were so variable under apparently similar conditions, that it seemed there must be something variable in the organisms themselves, which was not altogether a function of their surroundings.

## DEMONSTRATION OF DISSOCIATION INTO "ROUGH" AND "SMOOTH" TYPES.

The possibility of the presence of "rough" and "smooth" types in streptococcal cultures similar in properties to those found by Arkwright (1921) in some members of the intestinal group of bacteria presented itself. The "smooth" types of *B. typhosus* and *B. paratyphosus* grow in "regular" colonies, easily emulsifiable and not liable to spontaneous agglutination, whereas the "rough" types grow in "irregular" colonies, not easily emulsifiable and tending to agglutinate in normal saline.

Broth cultures of several strains of streptococci, 8 hæmolytic and 2 non-hæmolytic, were plated. From the plates numerous colonies were picked to serum broth and incubated for twenty-four hours. From the tube showing the most even turbidity, and that with least, plates were again made. The most even and the most irregular colonies were picked to serum broth. These operations were repeated over a period of about four months before a definite difference could be relied upon to appear in every culture. But each strain worked with has finally yielded two definite types of organisms—"roughs" and "smooths."

### *Characters of "Rough" and "Smooth" Types.*

The "smooths" grow with even turbidity in broth, and form bluish translucent colonies with even outline and a very finely granular surface on agar. The "roughs" grow as a precipitate in broth, and on agar have white, more opaque, coarsely granular colonies with irregular outlines, often appearing as a tangled mass of one long chain of organisms. Microscopically the "roughs" show more clumps and much longer chains than the "smooths"; also *more variation in size* with a distinct tendency to be larger. Some of the "rough" forms also have a mucous growth in broth. No capsules have been demonstrated.

### *Hæmolysin Production.*

The power of the two types to produce hæmolysin was tested on sheep's blood-corpuscles, the broth cultures being centrifuged to prevent any action on the part of the organisms themselves, so far as possible. First results seemed to indicate that the "smooth" type was much more active in producing

hæmolysin than the "rough" type. However, it was observed that in a mucoid "rough" culture, when some strands of growth were suspended in the fluid, there was a greater amount of hæmolysin than if the growth were all in the bottom of the tube. The probability was that the active hæmolytic substance was closely allied to the organisms, and did not readily diffuse from a sediment growth as was found by Lyall (1914). To ascertain whether this was the case eighteen-hour cultures of "roughs" were tested to see whether a difference existed between the hæmolytic activity of the fluid carefully pipetted from the top of the cultures and of the fluid immediately surrounding the organisms. The difference was very marked, the upper layer giving no hæmolysis or partial in low dilutions while the lower layer gave complete hæmolysis in varying dilutions.

The action of "roughs" and "smooths" on blood plates was also tested. Five strains were plated on 10 per cent. rabbit's blood agar. There was some variation in the size and completeness of the ring of hæmolysis around the colonies, but this depended on the size of the colony and not on whether it was "rough" or "smooth." The results were the same for three tests of the series.

Since obtaining these results all cultures to be tested for hæmolytic activity have been well shaken before centrifuging, and "roughs" and "smooths" have then been found to agree very closely in hæmolytic property.

TABLE I.—*Hæmolytic Activity.*

| Strains. | Dilutions of broth cultures with degree of hæmolysis. | | | | |
|---|---|---|---|---|---|
| | 1 : 2. | 1 : 4. | 1 : 8. | 1 : 20. | 1 : 40. |
| First results: | | | | | |
| "H. Cohen," rough | — | — | — | — | — |
| " smooth | C | C | C | AC | AC– |
| "2384," rough | AC | AC+ | Tr | — | — |
| " smooth | C | C | C | C | C |
| "Crowe," rough | C | AC | AC– | Tr | — |
| " smooth | C | C | C | C | C |
| Top and lower layer of rough cultures: | | | | | |
| "H. Cohen," R., top layer | C | C | AC | Tr+ | Tr |
| " R., lower layer | C | C | C | C | C |
| "2384," R., top layer | C | C | AC | Tr+ | Tr |
| " R., lower layer | C | C | C | C | C |
| "Crowe," R., top layer | — | — | — | — | — |
| " R., lower layer | C | C | C | Tr+ | Tr+ |
| Estimations after shaking: | | | | | |
| "H. Cohen," rough | C | C | C | C | C |
| " smooth | C | C | C | C | C |
| "2384," rough | C | C | C | C | Tr+ |
| " smooth | C | C | C | C | C |
| "Crowe," rough | C | C | C | Tr+ | Tr |
| " smooth | C | C | C | Tr+ | Tr |

C = Complete hæmolysis. Tr = Trace of hæmolysis.
AC = Almost complete hæmolysis. Tr+ = More than trace of hæmolysis.
AC– = Less than almost complete hæmolysis. – = No hæmolysis.

### *Virulence.*

*Preliminary observations.*—The virulence of the two types of the 8 hæmolytic strains was tested on mice and rabbits.

Eighteen-hour broth cultures were made. The hæmolytic activity of each was tested and 0·5 c.c. of each was injected intraperitoneally into mice, 2 mice being inoculated with each culture. The whole series was later repeated, so that in all 32 mice were given "roughs" and 32 mice were given "smooths."

At the end of 3 days after injection, 24 of the animals inoculated with "roughs" survived and only 4 of those which received "smooths." One of the "smooth" strains was apparently not virulent for mice, as the 4 given that strain survived. From the heart's blood of each mouse which died a culture of hæmolytic streptococci was isolated; the passage did not appreciably alter the "roughness" or "smoothness" of the type.

*There was no relation between the number of deaths and the amount of hæmolysin in the cultures.*

However, the "rough" and "smooth" types of the two non-hæmolytic strains injected into mice in duplicate in the same amount as the hæmolytic ones caused no deaths.

*Inoculation of rabbits.*—Twenty-four-hour growths on agar slopes of both types of the 8 hæmolytic strains were made. Each was emulsified in 1 c.c. of normal saline and injected *intravenously* into a rabbit. The results were as follows:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 3 | rabbits | injected | with | "smooth" | types | died in | 6 days. |
| 1 | rabbit | ,, | ,, | ,, | ,, | ,, 8 | ,, |
| 2 | rabbits | ,, | ,, | ,, | ,, | ,, 10 | ,, |
| 1 | rabbit | ,, | ,, | ,, | ,, | ,, 24 | ,, |
| 1 | ,, | ,, | ,, | "rough" | ,, | ,, 6 | ,, |

Thus 7 of the 8 inoculated with "roughs" survived and one only of the 8 inoculated with "smooths."

Two of the rabbits which died in 6 days showed small abscesses in the liver and very small ones in the kidney. The remaining rabbits had no apparent macroscopic lesions. From the heart's blood of each rabbit a pure culture of a hæmolytic streptococcus was isolated.

The surviving rabbits, viz. 1 injected with "smooths" and 7 injected with "roughs," were killed 4 weeks after injection. Cultures from the heart's blood and liver of each were sterile.

*Immunity experiments: Experiment I.*—The "rough" and "smooth" types belonging to 7 different strains had been inoculated subcutaneously into mice (in duplicate), with the result that, at the end of 6 days, 5 out of 14 inoculated with "smooths" survived and 9 out of 14 inoculated with "roughs." The difference in virulence as estimated by the subcutaneous route was apparently not so dramatic. It was decided to leave these 14 mice for a further period of 16 days in order to ascertain whether any immunity to the virulent "smooth" type had developed. After 16 days, however, the survivors were reduced to 9, viz. 7 which had received "roughs" and only 2 which had received "smooths." Each of these was given 0·5 c.c. of a 24-hour broth culture of the corresponding "smooth" intraperitoneally.

A corresponding number of untreated mice were inoculated as controls. Little immunity resulted, as at the end of 3 days only 1 mouse previously treated with "rough" was living. However, this may have been due to the fact that the dose was considerably above the minimal lethal dose.

*Experiment II.*—The "rough" and "smooth" types of 1 strain were given to mice (in duplicate) in quantities of 1 slope $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$ slope. Thus in all 12 received "rough" and 12 received "smooth."

In 3 days 7 inoculated with "smooth" were dead but none inoculated with "rough." The remaining mice were kept under observation for 10 days to give them the chance to develop protective bodies. At the end of 10 days only 10 of the 17 survived, viz. 10 of the original 12 mice inoculated with "roughs." These 10 were now given 0·5 c.c. of a "smooth" culture of the same strain. One control was also injected with this quantity which had previously been found to kill 4 out of 6 mice in 24 hours. The results were as follows: Two reinjected mice died overnight; control mouse died in 48 hours; 1 reinjected mouse died in 3 days.

Thus 7 out of 10 mice which had survived inoculation with "roughs" had undoubtedly acquired a very considerable degree of immunity to the corresponding "smooth" type.

*Examination of peritoneal exudate after inoculation.*—To discover, if possible, whether any difference could be detected in the behaviour of "roughs" and "smooths" after injection into the peritoneal cavity of mice, one mouse was given 0·5 c.c. of a 24-hour broth culture of "rough" and one the same amount of "smooth" of the same strain. Fluid was removed from the peritoneal cavity after 30 minutes, 1 hour, and then hourly up to 8 hours. Four strains were investigated in this way.

During the first two hours the "smooths" showed slight decrease or no change in numbers. A rapid increase then followed and 3 of the 4 mice died overnight. The remaining mouse recovered, and it was observed that in the case of this animal the organisms decreased from the fourth hour onwards. With regard to the "roughs" only a few cocci were present after 30 minutes. In one case the cocci remained fairly constant until the second hour, when a rapid decrease ensued.

In all cases lymphocytes and a very few polymorphonuclear leucocytes were found during the first hour; from the second hour the number of polymorphonuclear leucocytes increased and this increase continued during the period of examination. With the "smooths" the increase was much less than with the "roughs." Some phagocytosis was seen but never to any marked degree.

*Late examination of mice which had survived intraperitoneal inoculation of "rough" types.*—Mice injected intraperitoneally with "rough" types and which died or were killed four or six days or even weeks later showed abscesses in the abdominal cavity. These varied in size from that of a small pea to one including almost one whole lobe of the liver. The greatest number have been in the splenic omentum. Some occurred also on the surface of the liver, such sites suggesting almost certainly a purely local lymphatic spread from the peritoneal cavity. The abscesses were walled off. From each abscess a "rough" growing hæmolytic streptococcus was isolated. In two mice which were killed 8–10 days after inoculation with "smooths," small abscesses were also found in the peritoneal cavity, and from these cultures of hæmolytic streptococci which were a mixture of "rough" and "smooth" were grown. A few mice which survived a "smooth" injection for six days and then died

showed no abscesses, but a hæmolytic "smooth" streptococcus was isolated from the heart's blood.

TABLE II.—*Virulence.*

| Strain. | Dilution of culture giving complete hæmolysis. | Animals injected. | Dose. | Death or survival. |
|---|---|---|---|---|
| First series: | | | | |
| "2384," rough | 1 : 20 | 2 mice | 0·5 c.c. 24-hr. broth culture | *Both alive on 7th day.* |
| „ smooth | 1 : 20 | 2 „ | Do. | † 24 hrs. † 48 hrs. |
| "W.," rough | 1 : 8 | 2 „ | „ | † 24 hrs. *Alive on 7th day.* |
| „ smooth | 1 : 8 | 2 „ | „ | † 24 hrs. † 48 hrs. |
| "L.," rough | 1 : 4 | 2 „ | „ | *Both alive on 7th day.* |
| „ smooth | 1 : 4 | 2 „ | „ | Both † 24 hrs. |
| "B.C.," rough | 1 : 40 | 2 „ | „ | *Both alive on 7th day.* |
| „ smooth | 1 : 40 | 2 „ | „ | Both † 24 hrs. |
| "Crowe," rough | 1 : 8 | 2 „ | „ | *Both alive on 7th day.* |
| „ smooth | 1 : 8 | 2 „ | „ | Both † 24 hrs. |
| "2716," rough | 1 : 8 | 2 „ | „ | *Both alive on 7th day.* |
| „ smooth | 1 : 8 | 2 „ | „ | Both † 24 hrs. |
| "A.B.," rough | 1 : 8 | 2 „ | „ | *Both alive on 7th day.* |
| „ smooth | 1 : 8 | 2 „ | „ | „ |
| "H. Cohen," rough | 1 : 20 | 2 „ | „ | † 24 hrs. *Alive on 7th day.* |
| „ smooth | 1 : 20 | 2 „ | „ | Both † 24 hrs. |
| Second series: | | | | |
| "2384," rough | 1 : 20 | 2 „ | „ | † 6 days. *Alive on 7th day.* |
| „ smooth | 1 : 40 | 2 „ | „ | Both † 24 hrs. |
| "W.," rough | 1 : 40 | 2 „ | „ | † 24 hrs. and † 6 days. |
| „ smooth | 1 : 40 | 2 „ | „ | Both † 24 hrs. |
| "L.," rough | 1 : 8 | 2 „ | „ | *Both alive on 7th day.* |
| „ smooth | 1 : 8 | 2 „ | „ | Both † 24 hrs. |
| "B.C.," rough | 1 : 40 | 2 „ | „ | *Both alive on 7th day.* |
| „ smooth | 1 : 40 | 2 „ | „ | Both † 24 hrs. |
| "Crowe," rough | 1 : 20 | 2 „ | „ | † 48 hrs. and † 72 hrs. |
| „ smooth | 1 : 20 | 2 „ | „ | Both † 24 hrs. |
| "2716," rough | 1 : 20 | 2 „ | „ | † 6 days. *Alive on 7th day.* |
| „ smooth | 1 : 20 | 2 „ | „ | Both † 48 hrs. |
| "A.B.," rough | 1 : 20 | 2 „ | „ | † 48 hrs. *Alive on 7th day.* |
| „ smooth | 1 : 20 | 2 „ | „ | *Both alive on 7th day.* |
| "H. Cohen," rough | 1 : 40 | 2 „ | „ | Both † 24 hrs. |
| „ smooth | 1 : 40 | 2 „ | „ | „ |
| "R.," rough | non-hæm. | 2 „ | „ | *Both alive on 7th day.* |
| „ smooth | „ | 2 „ | „ | „ |
| "Infrequens," rough | „ | 2 „ | „ | „ |
| „ smooth | „ | 2 „ | „ | „ |
| "2384," rough | — | 1 rabbit (900 grm.) | 1-24-hr. slope | † 10 days. No appareut lesions. |
| „ smooth | — | 1 rabbit (900 grm.) | „ | † 6 days. No apparent lesions. |
| "W.," rough | — | 1 rabbit (1100 grm.) | „ | *Survived.* |
| „ smooth | — | 1 rabbit (1120 grm.) | „ | † 24 days. No apparent lesions. |
| "L.," rough | — | 1 rabbit (800 grm.) | „ | *Survived.* |
| „ smooth | — | 1 rabbit (900 grm.) | „ | † 6 days. Slight gelatinous exudate around heart. |

TABLE II.—*Virulence*—continued.

| Strain. | Dilution of culture giving complete hæmolysis. | Animals injected. | Dose. | Death or survival. |
|---|---|---|---|---|
| Second series—*continued*: | | | | |
| "B.C.," rough | — | 1 rabbit (1200 grm.) | 1-24-hr. slope | *Survived.* |
| „ smooth | — | 1 rabbit (1400 grm.) | „ | † 10 days. No apparent lesions. |
| "Crowe," rough | — | 1 rabbit (1000 grm.) | „ | *Survived.* |
| „ smooth | — | 1 rabbit (1020 grm.) | „ | † 8 days. No apparent lesions. |
| "2716," rough | — | 1 rabbit (1280 grm.) | „ | *Survived.* |
| „ smooth | — | 1 rabbit (1350 grm.) | „ | † 6 days. Small abscesses in liver and kidney. |
| "A.B.," rough | — | 1 rabbit (1150 grm.) | „ | *Survived.* |
| „ smooth | — | 1 rabbit (1150 grm.) | „ | *Survived.* |
| "H. Cohen," rough | — | 1 rabbit (1200 grm.) | „ | *Survived.* |
| „ smooth | — | 1 rabbit (1220 grm.) | „ | † 6 days. Small abscesses on kidneys. |

† = Death. Mice inoculated intraperitoneally. Rabbits inoculated intravenously.

## DISCUSSION.

The presence of "roughs" and "smooths" in streptococcal cultures would account for many of the difficulties encountered in agglutination work because of the fact that the "roughs" grow in clumps, and it remains to be determined now whether these types are distinguishable serologically. This question is being considered.

The mechanical or physical factor in the diffusion of the hæmolysin would account for decrease in the hæmolytic activity of a culture following an increased proliferation of the "rough" elements.

The most striking outcome, however, of these researches is the demonstration of an almost complete lack of virulence on the part of the "rough" elements in streptococcal cultures. Accurate knowledge has hitherto been wanting on this highly important association of virulence differences with colony form, but it is of much interest that de Kruif (1921) has recorded a very similar association in connection with a strain of *Pasteurella*.

So far as one may judge from the intra-peritoneal inoculations in mice, it would seem that the mechanical arrangement of the "roughs" in clumps might go far to explain the tardy and only very local proliferation in tucked-away corners of omental tissue, the consequent abeyance of blood invasion, and the final disposal of the infecting "roughs" in localised walled-off foci through the agency of the peritoneal lymphatics.

The clumps are in fact dealt with exactly like inorganic foreign particulate matter such as melanin when introduced into the peritoneal cavity (Ledingham, 1909). The ultimate fate of these localised foci is yet uncertain, but it is of

considerable interest that their presence is associated with a definite acquired immunity to the corresponding virulent " smooth " type.

While the sequence of events after intraperitoneal inoculation of "roughs" in the mouse took this particular form, there was no evidence that rabbits which survived *intravenous* inoculation with " roughs " harboured such foci when later submitted to autopsy. The mechanical factor, therefore, will only partially explain the lack of virulence or invasive power.

Finally, a consideration of the data relating to degree of hæmolytic activity and associated virulence (detailed in Table II) would indicate that hæmolysin-production has not the close relationship with virulence which many workers have alleged, and the results in this respect are in agreement with those of Lyall (1914), Longcope, Stevens and Brady (1920), and Stevens, Brady and West, (1921) whose work lends no support to any such intimate relationship.

## SUMMARY.

(1) Individual strains of streptococci can be "dissociated " into " rough " and " smooth " types which show remarkable differences in virulence for laboratory animals.

(2) Inoculation with the " rough " or avirulent type appears to afford considerable protection against the " smooth " or highly virulent form.

(3) Hæmolysin production is not a dominating factor in streptococcal virulence.

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I wish to thank Dr. Ledingham for much helpful advice and criticism in the course of this work.

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# EXPERIMENTS ON THE INFECTIVITY OF TYPHUS VIRUS CONTAINED IN LICE (*PEDICULUS HUMANUS* AND *PEDICINUS LONGICEPS*).

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THE experiments detailed in this paper were performed with two distinct strains of typhus virus. The first strain used, for which our thanks are due to the League of Red Cross Societies Typhus Research Committee, was brought from Warsaw by one of us in the summer of 1920, inoculated in guinea-pigs.

After a number of passages through guinea-pigs, a monkey and human lice, this virus died out, whether from attenuation or not it is difficult to say. Through the kindness of Prof. A. Stokes, of Dublin, we obtained some guinea-pigs inoculated with the blood of a typhus patient from Ireland.

The monkeys used were *Macacus rhesus* except one, which was a *Macacus* of another species (unidentified).

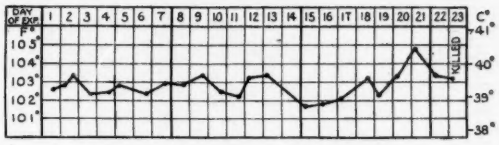
## THE INFECTION OF GUINEA-PIGS BY THE INJECTION OF INFECTED HUMAN LICE.

In common with previous workers we found no difficulty in infecting guinea-pigs with emulsions in salt solution of human lice which showed a heavy infection with *Rickettsia prowazeki*. The dose of virus, even when only one or two lice are used, is large in comparison with guinea-pig to guinea-pig injection, judging by the early reactions of temperature in the animal, in some cases as early as the fifth or sixth day.

Two methods of infecting the human lice were employed in the experiments detailed in this and other sections of the paper: (1) By feeding the insects on a typhus-infected monkey, and (2) by the rectal injection (Bacot, 1922) of virus obtained by centrifuging blood of typhus-infected guinea-pigs or the emulsified alimentary system of a previously infected louse.

*Experiment* 1.—The alimentary tracts of two living human lice which had fed on a typhus-infected monkey for 8–11 days were emulsified in salt solution and injected into the peritoneal cavity of guinea-pig No. 38.

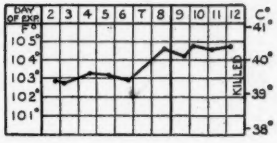
The heart blood of this guinea-pig infected, on inoculation, another, which showed typical brain lesions. The temperature reaction of this guinea-pig was exceptionally late (20th–21st day).




Temperature Chart, Guinea-pig No. 38.

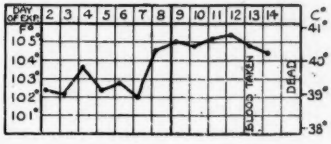
*Experiment* 2.—The alimentary tracts of 3 human lice which had fed on a typhus-infected monkey were emulsified in salt solution and injected into the peritoneal cavity of guinea-pig No. 44.

Sections of the brain showed typical lesions, and the blood and brain infected other guinea-pigs.




Temperature Chart, Guinea pig No. 44.

*Experiment* 3.—The alimentary tracts of 7 human lice, which had been infected by the injection, *per rectum*, of an emulsion of the alimentary system of another infected louse, were injected subcutaneously into guinea-pig No. 148. Six of these lice had died in an interval of two days preceding the injection, but the seventh survived. They constituted the fourth passage through lice from the original batch which had been injected with platelet material* from the blood of a typhus-infected guinea-pig.




Temperature Chart, Guinea-pig No. 148.

## FAILURE TO INFECT MONKEYS OR A GUINEA-PIG BY BITES OF INFECTED HUMAN LICE.

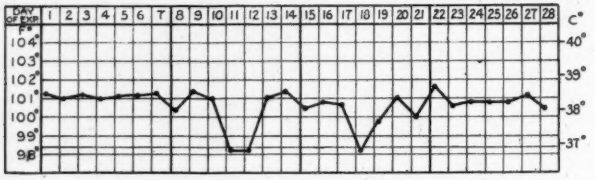
The infected lice were fed on an infected area of skin that had been shaved on the previous day. All possible care was taken to avoid contamination of the animal's skin with excreta from the lice, which were closely watched while feeding, and any fæces passed during the process were, if possible, removed before coming into contact with the skin. In the few instances where contact was unavoidable the excreta were removed at once, and the spot on which they had fallen was sponged over with a minute swab of cotton-wool soaked with 2 per cent lysol. Between the intervals of feeding the lice were kept in an incubator at a temperature of about 32° C. (90° F.).

*Experiment* 4.—Six human lice infected by the rectal injection of platelet material on 30 . ix . 21 and ten that had been similarly infected on 5 . x . 21 were fed by the rectal injection of human blood until 7 . x . 21, when they were given one feed upon monkey No. 4, and subsequently twice daily for five days. During this period the insects were dying off rapidly and only two survivors

* We are indebted for this material to Dr. Ségal.

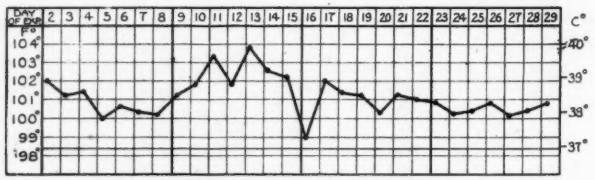
lived to feed on the fifth day. All the lice which died showed *Rickettsia prowazeki* on microscopic examination, and as the two survivors were too feeble to feed after the fifth day their emulsified alimentary tracts were inoculated into two guinea-pigs, both of which developed typhus.

Monkey No. 4, however, developed no fever.



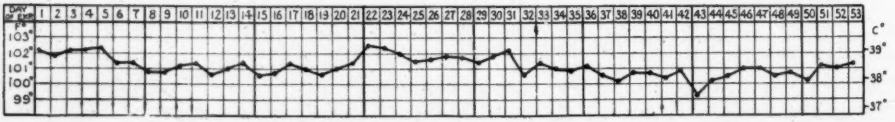

**Temperature Chart, Monkey No. 4 (1st).**

Half the brain of an infected guinea-pig was injected subcutaneously into this monkey a month later and produced a well-marked typical attack of fever in the second week, showing that the monkey was not immune and that the lice in all probability had failed to infect it.



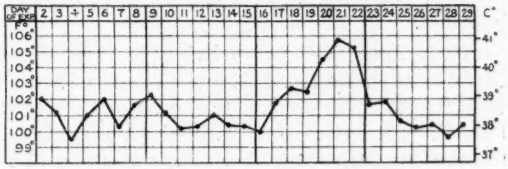

**Temperature Chart, Monkey No. 4 (2nd).**

*Experiment* 5.—Twenty-three human lice which had been infected by feeding on a typhus-infected monkey (No. 4) twice daily for a week, commencing at the period when its temperature was at its height, were fed twice daily on monkey No. 5. Their numbers, however, rapidly dwindled until there were only three living on the 5th and last day of feeding. Smears made from two of the three showed *Rickettsia prowazeki* in an early phase of development. There is little doubt that these lice contained the virus of the disease, yet the monkey showed no rise of temperature.



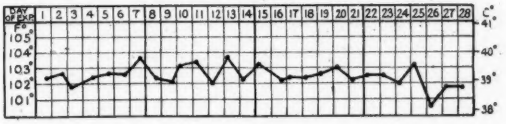

**Temperature Chart, Monkey No. 5 (1st).**

A test dose of virus, half a brain of an infected guinea-pig, was injected subcutaneously into this monkey about two months after the beginning of the experiment and produced a well-marked temperature reaction, proving that the animal was not immune.



Temperature Chart, Monkey No. 5 (2nd).

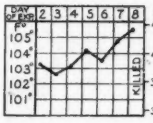
*Experiment* 6.—Fourteen human lice which had been infected by feeding on a typhus-infected monkey (No. 1) twice daily for 10–13 days were given one feed on guinea-pig No. 43.



Temperature Chart, Guinea-pig No. 43 (1st).

This guinea-pig was subsequently infected by the injection of a portion of brain of an infected guinea-pig, proving it not to be immune.

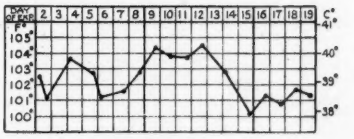
Of the 14 lice fed upon it 12 fed fully, one fed slightly, and one did not obtain any blood. Smears made from these lice subsequently showed that nearly all were infected with *Rickettsia prowazeki*, most of them heavily, and an emulsion of three of them produced typhus in guinea-pig No. 44 referred to above (Experiment 2).



Temperature Chart, Guinea-pig No. 43 (2nd).

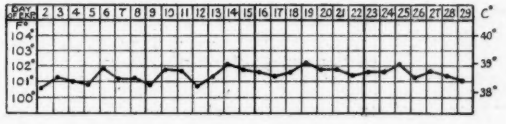
## THE INFECTION OF MONKEYS AND ATTEMPTED INFECTION OF GUINEA-PIGS BY THE VIRUS CONTAINED IN INFECTED MONKEY LICE (*PEDICINUS LONGICEPS*).

*Experiment* 7.—The alimentary canals dissected out of 36 monkey lice were emulsified in salt solution and injected subcutaneously into the thigh of monkey No. 2. These monkey lice, with others, were captured on a typhus-infected monkey (No. 1) on the 12th day after its initial rise of temperature in response to the injection of infected guinea-pig brain. Smears made from the emulsion injected into monkey No. 2 as well as smears made from individual specimens of the captured lice showed *Rickettsia prowazeki*.



Temperature Chart, Monkey No. 2 (1st).

Eight months later this monkey received an injection of half a brain of an



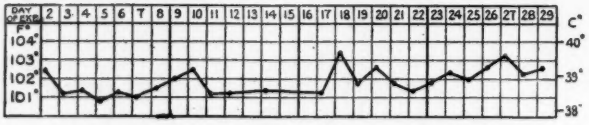
Temperature Chart, Monkey No. 2 (2nd).

infected guinea-pig, but there was no rise of temperature, showing that it was immune.

*Experiment* 8.—Seven living monkey lice were transferred from monkey No. 2 to monkey No. 3 at a time when they might be expected to be infected with the typhus virus. This monkey's temperature was taken for 26 days but did not rise. The animal died on the 13th day after we had ceased to take its temperature. A large number of monkey lice were collected from the body, and a microscopic examination of smears made from 46 of these lice showed 31 of them to be infected with *Rickettsia prowazeki*.

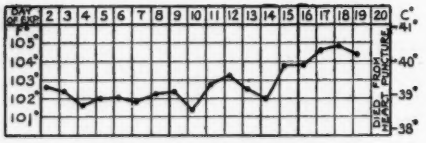
Arkwright, Bacot and Duncan (1919) have recorded that they examined normal monkey lice and found them free from *Rickettsia*. One of us (B.), when in Warsaw, examined a further series, and in the course of this work we have examined yet another series. In all, smears made from 150 *Pedicinus longiceps* taken from normal monkeys have been examined without any traces of *Rickettsia* bodies being discovered. We therefore assume on the strength of the presence of *Rickettsia prowazeki* in the lice taken from this monkey after its death that it developed an attack of typhus subsequent to our lapse in recording its temperature.

*Experiment* 9.—An emulsion of 100 monkey lice taken from monkey No. 3 (Experiment 8) was injected into the peritoneal cavity of guinea-pig No. 65, but no rise of temperature indicating typhus occurred.



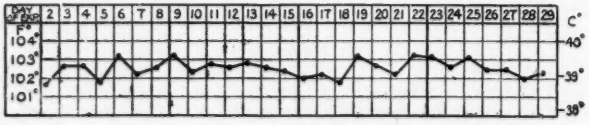

Temperature Chart, Guinea-pig No. 65 (1st).

Ten months later a quarter of a typhus-infected guinea-pig brain injected into the peritoneal cavity produced typical fever, showing that the animal was not immune.



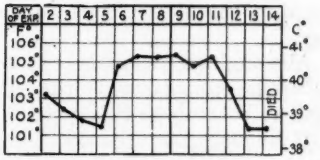

Temperature Chart, Guinea-pig No. 65 (2nd).

*Experiment* 10.—The alimentary tracts of five monkey lice, taken from monkey No. 4 (Experiment 4) about a fortnight after the height of its fever, were emulsified in salt solution and injected subcutaneously into guinea-pig No. 147 without producing any rise of temperature. A smear of the emulsified guts showed the presence of *R. prowazeki*.




Temperature Chart, Guinea-pig No. 147 (1st).

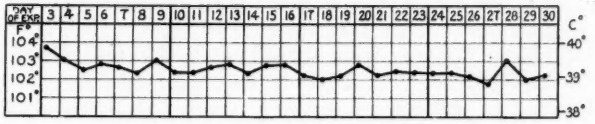
Seven weeks later a quarter of a typhus guinea-pig brain produced the usual well-marked rise of temperature.




Temperature Chart, Guinea-pig No. 147 (2nd).

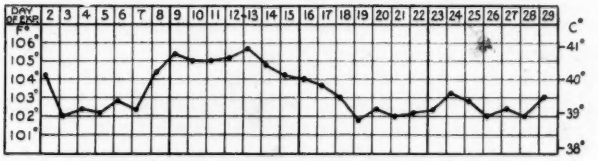
## ATTEMPT TO INFECT A GUINEA-PIG WITH THE EXCRETA OF TYPHUS-INFECTED HUMAN LICE.

*Experiment* 11.—The excreta used for this experiment were collected on two consecutive days from the lice fed on monkey No. 4. They were collected as they were ejected by the insect with a fine sable-haired paint brush moistened with salt solution and were put in a small tube containing a dish of salt solution. Those collected at the evening feed were placed in the same tube as those collected in the morning, each day's collection being injected into the guinea-pig the evening of the day on which it had been collected. Three of the lice fed on monkey No. 4 (which probably contributed to the excreta used for inoculation) were emulsified and inoculated into three guinea-pigs. One died some days after the injection with a high temperature; the other two both showed typical typhus charts. The two injections of excreta produced no rise of temperature.




Temperature Chart, Guinea-pig No. 131 (1st).

Subsequently this guinea-pig received an injection of a quarter of a brain of a typhus-infected guinea-pig. Its temperature reaction in the chart below shows that it was not an immune animal.




Temperature Chart, Guinea-pig No. 131 (2nd).

## COMMENTS.

As noted by previous workers, who have compared strains of typhus virus from various countries, we found that the Polish and Irish strains were apparently identical. Guinea-pigs which had survived attacks of typhus

caused by inoculation with the Polish strain proved to be immune to the Irish virus.

Although our experiments were not planned with a view to determining if *Rickettsia prowazeki* is the causal organism of typhus, all the evidence obtained bearing on this question tends to support the view that it is so.

It seems rather remarkable that while there was no difficulty in infecting monkeys with typhus through the medium of monkey lice (if the evidence in the case of monkey No. 3 in Experiment No. 8 be admitted, it was a case of natural transmission), yet all three attempts to transmit the disease to guinea-pigs by the injection of emulsified *Pedicinus longiceps* taken from the infected monkeys failed. Unless this failure be attributed to mere chance it would seem to suggest a lowered virulence in the virus when passed through monkey lice.

More definite is our failure in the case of both monkeys and a guinea-pig to infect by bite. Although the number of trials were few they appeared to be favourably circumstanced, except, of course, that the lice were not feeding on their natural host, but this, it will be remembered, has proved no bar to insect transmission by bite in the case of other diseases. The only other anomaly which is of some importance as a negative result was obtained, is that in the case of the monkey experiments the lice used were not infected in the natural way by feeding on an infected animal, but by rectal injection of typhus virus.

The evidence obtained from smears made from the salivary glands carefully dissected out of a few lice heavily infected with *Rickettsia prowazeki* tends to negative any suggestion of the transmission of typhus by the medium of the salivary fluid. In no instance were smears made of the large reniform glands, which are normally free from contact with the stomach infected. More suspicion attached to the forked vermiform salivary glands which lie on the top of the stomach, but the difficulty of avoiding contamination from the heavily infected cells of the stomach to the external surface of which the glands are attached by tracheal tubes, is very great. The most favourable method was found to be to cut off the fore end of the stomach after dissecting out the alimentary tract, remove it to a clean drop of salt solution, and gradually tease away fragments of the stomach from the attached glands; it was seldom found possible to obtain both glands free from injury. When freed from the stomach the glands were passed through clean salt solution and then teased. The numbers of *Rickettsia prowazeki* observed in these smears were few, if any, and in any case were infinitesimal compared with the numbers present in the smear of the drop of salt solution in which the operation of separating the glands from the fragment of stomach took place. The obvious conclusion was that the vermiform salivary glands were not infected, the number of *R. prowazeki* observed being more reasonably accounted for on the supposition that they were carried over from the heavily infected stomach-wall.

## RÉSUME.

(1) A Polish and an Irish strain of typhus virus appeared to be identical, as shown by cross-immunity experiments.

(2) Guinea-pigs were infected by the injection of the emulsified alimentary tracts of human lice, which showed heavy infection with *Rickettsia prowazeki*. Some of these lice had been infected by feeding on typhus-infected monkeys, others by the rectal injection of platelet material obtained by the centrifugalisation of blood from infected guinea-pigs.

(3) One monkey was infected with typhus by subcutaneous inoculations of infected monkey lice (*Pedicinus longiceps*), and a second was probably infected by the transference of living monkey lice from an infected monkey.

(4) Two attempts to infect guinea-pigs by the injection of infected monkey lice failed.

(5) Attempts to infect monkeys and a guinea-pig by feeding infected human lice upon them failed.

(6) An attempt to infect a guinea-pig with the excreta taken from heavily infected human lice failed.

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# THE RELATION OF NATURAL DIPHTHERIA ANTITOXIN IN THE BLOOD OF MAN TO PREVIOUS INFECTION WITH DIPHTHERIA BACILLI.

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It has always seemed likely to some that natural diphtheria antitoxin appears in man as a result of previous infection with diphtheria bacilli. However, it has been very hard to prove this hypothesis. The Schick test has rather added to the difficulty, because the number of individuals who are Schick negative is too great to be accounted for by previous attacks of diphtheria. And again, it is observed in many cases that an actual attack of diphtheria produces no natural antitoxin subsequently. The suggestion may be made that the antitoxin might be due to previous "carrier" infection, but this explanation also becomes unsatisfactory in view of the universal experience of all workers that virulent "carriers" have negative Schick reactions, *i. e.*, are already immune, the Schick positive healthy "carrier" being unknown.

Owing to a fortunate combination of circumstances, some experimental Schick tests carried out at a residential school seem capable of throwing light on the subject, and supporting the reasonable view that immunity to diphtheria is caused by recognised or unrecognised contact with diphtheria bacilli.

## MATERIAL AND TECHNIQUE.

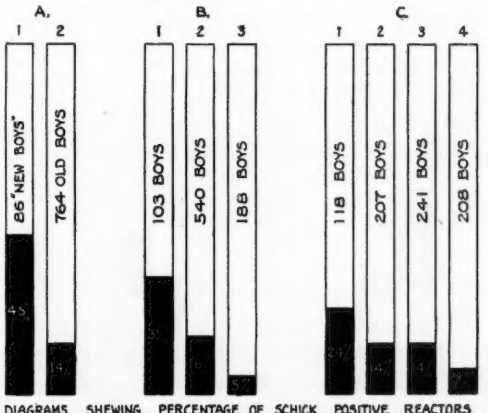
The school consists of 950 boys ranging from 11 to 16 years of age. They are all as physically perfect as possible, being selected by a strict medical examination with a a view to ultimate service in the Royal Navy. It should be noted, in particular, that special care is taken of the teeth, noses and throats of these boys. On the whole they are of a social grade higher than the ordinary Council schoolboy, and therefore, according to most observers, more susceptible to diphtheria—that is, they should contain among them a higher proportion of Schick positive reactors. Diphtheria has been epidemic in the school from May, 1921, to the present time. The first series of Schick reactions were all performed in February and March, 1922, and my thanks are due to Dr. A. T. MacConkey and Dr. G. F. Petrie of the Lister Institute, who supplied and re-tested the necessary undiluted toxin. The same batch of toxin was used throughout, and when tested at the end of the investigation the m.l.d.

remained the same. The usual technique was employed; one-fiftieth of a m.l.d. in 0·2 c.c. of saline *without phenol* was the dose used throughout. A control injection was always made on the other arm with the same diluted toxin heated. After each batch of tests a control test was performed on a known Schick positive reactor, always using a much smaller dose than that used on the tested schoolboys. The result was always a typical positive reaction.

## SCHICK IMMUNITY AND DIPHTHERIA PREVALENCE.

In the first series of tests 831 boys were examined. They can be divided into two groups: "new boys" who had not mixed with those already in the school, and "old boys" already in the school.

Diagram A, Fig. 1, shows the proportion of Schick positive reactors in these two groups. Nineteen new boys who joined in May, 1922, have been added to the original group of 67 "new boys" (Fig. 2) in order to increase the size of the sample. It is seen that 45 per cent. of 86 new boys were Schick positive reactors as against 14 per cent. of the boys already in the school—that is to say, the former contained proportionately three times as many boys susceptible to diphtheria as the latter. This difference is too great to be accounted for by the smallness of the sample of "new boys" or the difference in age. As regards age, among 19 new boys, aged 13 to 14 years, 8 (or 42 per cent.) were Schick positive reactors as compared with 14 per cent. of the same age among the "old boys." This difference in the percentage of non-immunes before and after joining the school at once suggested that the degree of immunity might be proportionate to the length of residence in the school.




DIAGRAMS SHEWING PERCENTAGE OF SCHICK POSITIVE REACTORS (SHADED BLACK) AMONG SELECTED GROUPS OF SCHOOL BOYS.

A. BEFORE & AFTER JOINING SCHOOL. (1) NEW BOYS BEFORE MIXING WITH (2) BOYS ALREADY IN SCHOOL.

B. AMOUNT OF EXPOSURE TO DIPHTHERIA. (1) LESS THAN 3 MONTHS. (2) TO ONE EPIDEMIC ABOUT 7 MONTHS (3) TO 2 EPIDEMICS, TOTAL ABOUT 10 MONTHS EXPOSURE TO INFECTION.

C. ACCORDING TO AGE (1) UNDER 13. (2) 13-14. (3) 14-15. (4) UNDER 15 YEARS OLD

FIG. 1.

It was possible by searching the school register to arrange the 831 Schick-tested boys in the order in which they had joined the school. A graph (Fig. 2). was then made as follows: The boys were divided into the batches who joined each term from September, 1917, when the most senior boys had joined the establishment. The fourteen terms since that date are written along the top of the graph; the number of boys joining at each of these periods is indicated by the figures along the bottom of the graph, and the graph itself is made by plotting out the percentage of Schick negative reactors among each

of the 14 samples of boys. The continuous line is a smoothed curve of the 14 points of reference thus obtained. This curve shows that the boys entering the school were under 60 per cent. immune according to their Schick reactions; boys with anything between six months' and two years' residence were 85 per cent. immune, while the oldest inhabitants, who had been $2\frac{1}{2}$ to $4\frac{1}{2}$ years at the school, were 95 per cent. immune to diphtheria. Now these alterations in degree of immunity occur by sudden steps, and not gradually, as they should if the immunising influence had been working throughout the whole period, and the degree of immunity was proportionate to the length of residence or age of the schoolboys. The shape of this curve—two steps connected by a horizontal line—is inexplicable until the diphtheria prevalence during the period under consideration is superimposed on the chart. The black rectangles on the bottom line indicate the duration and density of diphtheria infection as represented by the number of boys in whom bacilli morphologically resembling diphtheria bacilli were recorded as found. The school records show that there were 65 cases of diphtheria infection in the January to May term of 1919, after which, with the exception of an odd sporadic case or two, there is no entry until the start of the present outbreak, which commenced in May, 1921, reached its maximum in November, 1921, and has continued up to the present time. The diphtheria outbreaks are seen to coincide exactly with the steps in the graph, showing that immunising influences were working most rapidly in the presence of diphtheria. However, perhaps the most interesting feature is the horizontal line connecting the two steps, since it suggests that no immunisation of boys was taking place during the two years in which diphtheria was absent, because the sample of boys who entered the school in May, 1919, contains no more immunes than those who joined two years later in May, 1921, while those who joined before May, 1919, though only a few months longer in the school, contain 10 per cent. more Schick negative reactors. These senior batches had experienced an extra term of exposure to diphtheria infection.

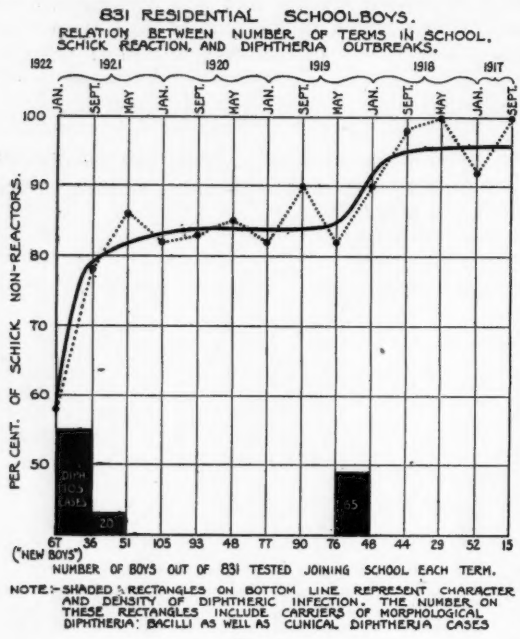



FIG 2.

Diagram B (Fig. 1) makes the relation between the schoolboys' Schick

reactions and diphtheria prevalence plainer. In the first column the black portion is proportionate to those boys who had been exposed less than three months to diphtheria ; they contained 35 per cent. Schick positive reactors. The second column represents the boys who had passed through seven months of the present diphtheria epidemic; they were 16 per cent. Schick susceptible, whereas the most senior group who had been through both epidemics, corresponding to a total of ten months' exposure to the risk of diphtheria infection, only contained 5 per cent. of non-immune boys. These diagrams do not, however, indicate the most important point, namely that no immunisation occurred in the absence of diphtheria, as is shown by the horizontal line in the graph.

Diagram C (Fig. 1) represents the 764 "old boys" arranged in four age-groups. The boys enter the school at from 11 to 14 years old but never remain after they are 16. If the appearance of natural antitoxin is a function of age in itself as distinct from a greater chance of infection, this diagram would show a regular series of steps from the youngest to the oldest boys. This, however, is not the case, as the two middle groups show no difference in immunity in spite of each of the samples exceeding 200. This diagram is merely the graph turned upside down. The youngest and oldest groups contain most of the junior and senior boys respectively, while the two middle groups comprise most of the boys who entered the school during the inter-epidemic period. Thus the age diagram still further confirms the deductions from the graph.

Assuming it is legitimate to apply the above figures generally, they mean that 45 out of every 100 boys who enter the school are Schick positive reactors, but on exposure to ten months of a diphtherial environment, such as existed in this school, 40 will become Schick negative reactors, in most cases without getting clinical signs of diphtheria—that is, 90 per cent. of positive reactors become negative reactors in ten months. Therefore a number somewhere in the neighbourhood of 27 per cent. should be found to change after three months' exposure to the same average concentration of diphtherial infection. To see if this reasoning was correct 100 unselected Schick positive reactors were re-tested after twelve to fourteen weeks had elapsed since their first tests. On the re-test 32 of these boys were found to have become negative reactors. Twenty-eight of these boys, who within three months had developed sufficient natural antitoxin to inhibit a Schick response, had no known history of diphtheria, but diphtheria cases and carriers were being recorded in the school all through the period between the two sets of tests. As it was very important that there should be no doubt that this result was not due to deterioration of the Schick toxin used, at the end of the re-test an injection of a two-thousandth of a m.l.d. (*i. e.* one-fortieth the Schick dose) was made into a known positive reactor with double the usual dose of heated toxin as a control, the same test solutions as those used on the re-tested boys being used. The usual typical positive reaction resulted, without any effect at the site of the control puncture. Another sample of boys consisting of 73 negative and 14 positive reactors were also re-tested thirty-four days after their first tests. None of the negative reactors had positive reactions, but two of the positive reactors had developed antitoxin within the month, becoming Schick negative. Thus the change in

these re-tests was always in the same direction—from positive to negative—never from immune to non-immune. In this way direct experiment proved that in three months 32 per cent. positive reactors had become negative in the presence of diphtheria, which approaches nearly to the 27 per cent. deduced from the graph (Fig. 2) drawn before the re-test was contemplated.

Recognised infection, that is, clinical diphtheria, according to the figures in this school, confers almost certain immunity. It is known that all diphtheria cases are Schick positive before attack. In 50 cases of clinical membranous diphtheria 42 were Schick negative and 8 positive reactors in from 3 to 6 months after therapeutic antitoxin had been given. Seven of the positive reactors were included in the re-tests mentioned above, 4 had become Schick immune, so that within nine months 92 per cent. of the post-diphtheritic cases had become Schick negative. Many observations in the literature show that a large proportion of post-diphtheritics have positive Schick reactions. Perhaps the high percentage which became Schick negative in this school is due to the fact that the convalescent diphtheria cases were returned into a diphtheria-saturated environment, where, if re-infected, they at once manufactured antitoxin instead of getting a second attack. This supposition is confirmed by the observation that no boy in this school had more than one attack of clinical diphtheria.

The missing link in the chain of evidence that natural antitoxin is due to infection with diphtheria bacilli is the Schick positive carrier of virulent bacilli. Over 200 throat cultures from healthy boys, who had been positive reactors at the time of the first series of tests, were examined. From 5 boys Neisser positive diphtheroids giving the fermentation reactions of *B. diphtheriæ* were obtained, but only one culture was toxigenic. It is a curious coincidence that 3 of these 5 carriers were found to have become Schick negative reactors at the re-test, including the one virulent carrier. This latter, whose Schick test was positive ten weeks previously but negative a fortnight subsequent to the discovery of the virulent bacillus, is the nearest approach to a true Schick positive carrier found in this school. One of the other two avirulent Schick positive carriers developed diphtheria subsequently with virulent bacilli in his throat similar in all other respects to the harmless organisms he had been known to carry for over two months previously, which shows that in this case, at any rate, avirulent diphtheria bacilli had had no influence in producing immunity.

During the period of this investigation the carrier-rate for "morphological" diphtheria bacilli, as estimated from fair (743) samples of the school population, was 3·5 per cent. But the virulent carrier-rate as calculated from the bacilli that were isolated was nearer 1 per cent. than 2 per cent. This seems to be a low figure, but in this population the chronic carrier was unknown, and the virulent, as distinct from the avirulent carrier, very rarely remained infectious over a second weekly examination. The very transitory nature of the virulent carrier state in this establishment suggests that a large proportion of boys became infected with a very low average rate. If we allow the average duration of carrying to have been ten days and the rate to have been 1 per cent., it would have been possible for 20 per cent. of the boys to have been infected as *recognisable* carriers during the seven months of the

diphtheria epidemic preceding the Schick examinations, and if we include the actual cases of diphtheria the figure would be about 30 per cent., which is more than sufficient to give every boy in the school the chance of coming into contact with diphtheria bacilli, especially when it is realised how closely together these boys sleep. There are over 100 boys to a dormitory, closely packed into six rows of beds.

## CONCLUSIONS.

In the school examined:

(1) "New" boys were three times as often susceptible to diphtheria as "old" boys.

(2) A graph is shown which indicates that the "old" boys became immune during outbreaks of diphtheria, and that between outbreaks no immunity was developed.

(3) The fact that the "old" boys were older was insufficient to account for the more frequent immunity.

(4) In the course of three months 32 per cent. of the susceptible boys became immune.

(5) In the course of nine months 92 per cent. of the boys who developed clinical diphtheria became immune.

(6) The period of "carrying" diphtheria bacilli was always short, and it is estimated that during seven months 30 per cent. of the boys were recognisable carriers.

(7) Under these circumstances it is probable that all boys in the school had the opportunity of being affected by the diphtheria bacillus to a slight unrecognisable degree.

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I wish to express my thanks to Surgeon-Captain P. M. May, R.N., and Surgeon-Commander S. Roach, R.N., for allowing me access to the school sick books. To the latter officer thanks are also due for his untiring work in arranging administrative details. Also acknowledgments are due to Surgeon-Lieutenant-Commander J. L. Priston, R.N., who helped with the Schick testing, and isolated many diphtheroids from the cultures I handed him.

# THE NATURE OF THE ACTION OF POTATO UPON THE GROWTH OF *B. INFLUENZÆ.*

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In a previous paper (Fildes, 1921) it was argued that the action of blood pigment in the growth of *B. influenzæ* was similar to its action upon guaiaconic acid in the presence of a peroxide. This argument was based upon the parallelism, among other things, which was shown to exist between the action of the pigments upon guaiaconic acid and upon the growth of the bacillus. It was concluded that the pigment operated as a "peroxidase" or catalyst in connection with the supply of oxygen to the bacillus.

Thjötta and Avery (1921),[2] testing the effect of other substances which contain peroxidases, have shown that *B. influenzæ* will grow satisfactorily in ordinary broth containing fragments of sterile potato. This result, according to these authors, is due to the occurrence in the potato of two factors similar to those which they and others have described in blood. One they name the "X" factor, which corresponds to the blood pigment, and the other the "V" factor, which is comparable to the non-pigmented fraction of blood-corpuscles (Fildes, 1921). They demonstrated that the reaction of these two factors in potato to heat is similar to the reaction of the comparable factors in blood, namely the "X" factor will withstand 120° C. for 45 minutes, while the "V" factor will only survive 100° C.

This resistance to heat of the potato X factor makes it difficult to suggest that the stimulating action of potato is due to a peroxidase operating in the same manner as the blood-pigment "peroxidase," because the vegetable peroxidases are stated to be "inactivated by heating in solution for a short time" (Falk, McGuire and Blount, 1919). Further, Thjötta and Avery observed that banana contained no peroxidase, but would grow *B. influenzæ*. They concluded that the "identity of the X factor must remain a matter of more or less conjecture. In blood this substance seems to be associated with the iron-containing pigment."

The work of Thjötta and Avery discounts the suggestion that a peroxidase is a dominant factor in the growth of *B. influenzæ*, and experiments were therefore carried out to check it.

## PARALLELISM BETWEEN THE HEAT-STABILITY OF POTATO X FACTOR AND POTATO PEROXIDASE.

The statement that banana contains no peroxidase was not confirmed, but the progressive growth in broth containing sterile potato was readily obtained. Their technique, however, of using a fragment of potato in a tube of broth did not appear well adapted for judging the effect of heat, and so a potato juice was prepared to replace the more complicated whole vegetable. Peeled potatoes were passed through a mincing machine and then through a press. In this way a brownish fluid heavily charged with starch grains and *débris* was obtained. This was diluted with an equal quantity of saline solution and filtered through paper pulp, a coarse "English" Berkefeld candle, and finally a Berkefeld N to sterilise.

### *Tests carried out in a slightly Alkaline Medium.*

The action of this potato juice, heated and unheated, upon the growth of *B. influenzæ* was then tested as follows:

*Experiment 1.*—Potato = fresh juice as above. Hæmatin = 2 per cent. solution, in a minimum of NaOH, of hæmin crystals separated from washed sheep's corpuscles by peptic digestion and washing; autoclaved. Yeast = watery extract of yeast according to Thjötta and Avery (1921).[1] Each tube contained 10 c.c. bactopeptone water; broth was not used because it contains small quantities of Thjötta and Avery's V factor. The reaction of the peptone water was pH 7·6–7·9, and it did not fall below pH 7·6 upon the addition of the slightly acid yeast and potato. The additions were made with calibrated dropping pipettes, thus: potato 1 c.c., yeast 0·1 c.c., hæmatin 0·025 c.c. The tubes containing potato were heated by immersion in boiling water for 20 minutes or autoclaving at 120° C. for the same time. All inoculations were made by adding 0·05 c.c. of an extremely thin suspension of *B. influenzæ* from a tube containing a minimum of blood, in saline solution, the strength being about 40,000 viable bacilli per c.c. The amount of growth was judged by opacity checked by microscopic examination and subculture with a standard loop holding 0·003 c.c. to prove purity. The opacity of the tubes containing heated potato was difficult to judge owing to the presence of a coagulum, and therefore a doubt is indicated in the protocols.

The strain of *B. influenzæ* used in this work was No. 291 of the *National Collection of Type Cultures*, London.

| | After 24 hours' incubation. | | |
|---|---|---|---|
| | Opacity. | Microscopic. | Subculture. No. of colonies. |
| Bactopeptone water: | | | |
| + Hæmatin | 0 | — | 0 |
| + Yeast | 0 | — | 0 |
| + Hæmatin + yeast | + | — | ∞ |
| + Potato, unheated | ++ | — | ∞ |
| + do. + hæmatin | ++ | — | ∞ |
| + do. + yeast | ++ | — | ∞ |
| + Potato, heated 100° C. | 0? | 0 | 0 |
| + do. + hæmatin | ++? | very numerous | ∞ |
| + do. + yeast | 0? | 0 | 0 |
| + Potato, heated 120° C. | 0? | 0 | 0 |
| + do. + hæmatin | 0? | 0 | 0 |
| + do. + yeast | 0? | 0 | 0 |

From this experiment it is clear that the X factor of potato is destroyed not only by heating to 120° C. but also to 100° C.—a result which is in marked contrast to that of Thjötta and Avery. On the other hand the V factor, as they state, survives the latter temperature.

To test the effect of heat upon the potato peroxidase the same materials were put through the same process together with the tubes destined for cultures. They were then cooled and tested by adding a freshly prepared solution of guaiaconic acid in alcohol and a few drops of ozonic ether. With unheated potato juice in peptone water a blue colour rapidly develops, but this is absent in both heated specimens. Thus there is, in fact, a parallelism between the X factor and the peroxidase in potato as close as between the same two factors in blood.

*Tests carried out in a Slightly Acid Medium.*

On considering further Thjötta and Avery's experiments, which gave results entirely in opposition to those here described, it appeared that they tested the heat stability by placing fragments of potato in broth, and thus the effect of heat might vary owing to the reaction of the substance of the potato being different. The reaction of the peptone water was pH 7·6–9, while the reactions of 12 different potato juices varied between pH 6 and 6·8. The previous experiment was, therefore, repeated, with the difference that the potato juice was heated in the slightly acid state *before* mixing with the peptone water.

*Experiment* 2.—Details as before. The first 6 tubes were as in Experiment 1 and are omitted.

| | After 24 hours' incubation. | | |
|---|---|---|---|
| | Opacity. | Microscopic. | Subculture. |
| Bactopeptone water: | | | |
| + Potato, heated 100° C. . . | ? | . numerous . | ∞ |
| + do. + hæmatin . . | ? + + | . very numerous | ∞ |
| + do. + yeast . . . | ? | . numerous . | ∞ |
| + Potato, heated 120° C. . . | ? | . scattered . | numerous |
| + do. + hæmatin . . | ? | . scattered . | numerous |
| + do. + yeast . . . | ? | . scattered . | ∞ |

In this experiment the X factor (as also the V factor) is shown to be much more resistant to heat as stated by Thjötta and Avery. It is probable from the results of the microscopic examination that at 120° a quantitative reduction in power has taken place, but this is far from complete.

Turning now to the parallel test of the peroxidase enzyme with guaiaconic acid, it is found that samples heated to 100° C. for 20 minutes in the slightly acid state are *not* deprived of peroxidase action. The colour development is slow but quite marked, particularly when the heated tubes are left standing overnight before testing. Under the same conditions a tube diluted with peptone water before heating shows no colour.

Specimens heated to 120° C. do not, however, show an obvious peroxidase reaction with guaiaconic acid, although, as previously stated, these stimulate the growth of *B. influenzæ*.

Thus at 100° C. there is still a parallelism between the survival of the X factor and the peroxidase in an acid medium, but this is not demonstrable at 120° C. It is, however, quite possible that the guaiaconic test is more gross than the growth test and that the latter might still give a positive result after the former had failed.

### PARALLELISM BETWEEN THE ABSORPTION OF POTATO X FACTOR AND POTATO PEROXIDASE.

In Experiment 2 it was noticed that the blue colour of the guaiaconic test was confined to the coagulum produced in the potato juice by heating. The following experiment was therefore carried out to show whether the coagulum differed from the supernatant fluid in content of X factor.

*Experiment* 3.—Details as before. The samples of potato juice were heated in duplicate, and one of each pair was then centrifuged to supply the supernatant. The first 6 tubes were as in Experiment 1 and are omitted.

| | After 24 hours' incubation. | | |
|---|---|---|---|
| | Opacity. | Microscopic. | Subculture. |
| Bactoteptone water: | | | |
| + Potato heated 100° C. supernatant | 0 | — | 0 |
| + do. + hæmatin | ++ | — | ∞ |
| + do. + yeast | 0 | — | 0 |
| + Potato, heated 100° C. deposit | ? | numerous | ∞ |
| + do. + hæmatin | ? | „ | ∞ |
| + do. + yeast | ? | „ | ∞ |
| + Potato, heated 120° C. supernatant | 0 | — | 1 |
| + do. + hæmatin | 0 | — | 100 |
| + do. + yeast | 0 | — | 0 |
| + Potato, heated 120° C. deposit | ? | isolated | very numerous |
| + do. + hæmatin | ? | „ | „ |
| + do. + yeast | ? | „ | ∞ |

From this experiment it is seen that the X factor is totally absent from the clear fluid after heating to 100° C., but is present in the coagulum. The same result is found after heating to 120° C. The guaiaconic test carried out on the same series shows that the peroxidase reacts in a similar manner. It is present in the deposit at 100° C., but not in the supernatant. As already stated, this test cannot be detected in the tubes heated to 120° C. It is probable that this absorption upon the coagulum has some bearing upon the resistance of the X factor and the peroxidase to heat (*cf.* Bayliss, 1918).

### DISCUSSION.

The demonstration of the effect of potato upon the growth of *B. influenzæ* appears to throw light upon the view previously advanced as to the nature of the effect of blood pigment. It is shown that a peroxidase-containing substance, namely the potato, can take the place of blood-pigment, although the essential properties in potato and blood-pigment are quite different.

The conspicuous point of similarity between the two is the possession of

the power to oxidise guaiaconic acid in the presence of a peroxide. In a previous paper it was shown that the peroxidase factor in blood ran parallel with the power of the blood to induce growth of the bacilli, and in this paper it is demonstrated that the totally different peroxidase in potato has an almost identical reaction to heat and absorption as has the growth-inducing property of potato.

It is therefore suggested that the growth-inducing property (Thjötta and Avery's X factor) is associated with the peroxidase enzyme, its function being to accelerate the transfer of oxygen to the bacillus.

## CONCLUSION.

It is suggested that the stimulating action of potato upon the growth of *B. influenzæ* is due to the action of the peroxidase enzyme operating in the same manner as blood-pigment to accelerate the transfer of atmospheric oxygen to the bacillus.

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# THE CULTIVATION OF *SPIRONEMA DUTTONI* FROM A CASE OF RELAPSING FEVER.

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THE spirochætes of relapsing fever were first cultivated by Noguchi (1912) from infected rats in his well-known tissue-ascites medium. He succeeded in propagating the different varieties for several generations. He found, however, that his cultures were viable only for a short time and that subcultures had to be made on the sixth or seventh day.

Starting with the Noguchi medium as basis, Kligler and Robertson (1922) developed a medium in which it was possible to cultivate consistently the spirochætes from rats, and in which the cultures remained viable for a period of three to seven weeks. The elements found to be essential for the growth and prolonged viability of these spirochætes were (*a*) a stabilised animal fluid having a reaction of pH 7·3, (*b*) a matrix of agar or fibrin, and (*c*) strict anaërobiosis. They observed that animal fluids (ascites, serum) become progressively alkaline on standing, and soon reach a reaction detrimental to the growth and life of the spirochætes, and that consequently the animal fluids used as substrates for the medium had to be neutralised and stabilised by means of some buffer.

On the basis of their observations these authors adopted the following medium: Horse or rabbit serum diluted 1 : 2 with saline, or undilated ascitic fluid, is adjusted to pH 7·0 and 1·0 per cent. peptone added (1·0 c.c. of a 10 per cent. peptone solution to 10 c.c. substrate). The medium is distributed in 3 to 4 c.c. amounts into Wassermann tubes. Each tube is inoculated with 0·1 c.c. infected blood, mixed and covered with a layer of paraffin 1·5 cm. high. In initial cultures the inoculated blood furnishes the fibrin "basket"; in subcultures 0·1 c.c. of fresh rabbit blood is added for that purpose. The tubes are kept for 24 hours at 37° C. and then at room temperature (about 20° C.).

The only report to my knowledge of direct cultivation of the relapsing fever spirochætes from the blood of a patient is that by Plotz (1917), using the Noguchi technique. The object of the paper is to describe the direct cultivation of the spirochætes from the blood of a case of relapsing fever by the method developed by Kligler and Robertson.

*History of the case.*—Before giving the details of cultivation it will be of interest to review briefly the history of the case because of its atypical character.

M. W., male, aged 30 years, admitted to hospital 20/5/21 with intermittent temperature. The temperature on admission at 12 mid-day was 37·6° C. At 12 p.m. it rose to 38·9° C., fluctuated for four days and returned to normal.

After two days it rose again, remained up for three days, dropped for one day, and rose again for the third time the day after. The pulse was rapid during the first attack but remained normal thereafter. Blood drops and smears examined on the 20th, 21st and 23rd were negative. The blood-count was normal except for the high percentage of large mononuclears (8 per cent.). On the 26th at 9 p.m. spirochætes were found in the drops but another examination at 8 a.m. on the 27th was negative. At 12 mid-day 30/5/21 spirochætes were again found both in the thick drops and in the dark field. Cultures as well as rat inoculations were thereupon made with positive results. At 4 p.m. 0·45 grm. salvarsan was injected intravenously; the temperature dropped immediately and remained normal until dismissal (June 6th).

*Cultures of Sp. duttoni (?).*—Cultures were made on 30/5/21 in buffered ascites and serum media and also in bouillon. On 6/6/21 actively growing cultures were found in the ascites and serum media. Successful subcultures were made on 15/6/21 and 2/7/21. On 20/7/21 the cultures were lost through a mould contamination. Three generations of spirochætes were thus obtained with successful transplants after sixteen and eighteen days respectively.

The cultures were characterised by the absence of any change in the medium. The upper portion of the fibrin-blood "basket" remained bright red; the fluid portion was clear and tinged by a slight hæmolysis. Most of the spirochætes were found in the fibrin and only few in the clear fluid. In the fibrin masses one could see clumps (colonies ?) as well as chains of two or three. If the culture remained clean and there was no marked hæmolysis, the spirochætes remained actively motile to the time of transfer. In some tubes degenerative changes characterised by rings on the spirochætes were observed. Any contamination promptly terminated the life of the spirochætes.

Rats inoculated with the second generation cultures, 2/7/21, became infected, and after forty-eight hours large numbers of spirochætes were found in the blood. The course of infection was the same as that produced with the patient's blood. Only one relapse was observed in the rats inoculated with culture material.

*Inoculation of rats.*—When the cultures were made, white rats were also inoculated intraperitoneally with the patient's blood. One to two days after the inoculation spirochætes appeared in the blood, increased in numbers for the next day or two and then disappeared, to reappear again after two days. No rat had more than one relapse. The strain was carried through three rat generations with the same results.

## CONCLUSION.

It would seem that with the method described it is possible to cultivate the spirochæte of relapsing fever from the blood of patients and maintain it under artificial cultivation for long periods.

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